PAS KINASE: ROLES IN REGULATING HEPATIC LIPID METABOLISM

by

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ABSTRACT

Excessive synthesis and storage of lipids is a prominent feature of the current epidemic of metabolic disorders, including obesity, diabetes and non-alcoholic fatty liver disease (NAFLD). Upon feeding, fatty acids and triglycerides are synthesized primarily in the liver in response to insulin signaling. This process is mediated by the sterol regulatory element binding protein 1c (SREBP-1c) transcription factor, a principal regulator of lipogenesis. Upon activation, SREBP-1c stimulates the transcription of the key lipogenic enzymes that catalyze the synthesis of fatty acids and their esterification to triacylglycerides. Hyperactivation of SREBP-1c has been implicated in promoting pathologic fat synthesis and driving features of the metabolic syndrome, including hepatic lipid accumulation (steatosis), dyslipidemia and insulin resistance.

PAS kinase (PASK) is an evolutionarily conserved serine/threonine kinase that has been proposed to function as a nutrient-responsive metabolic regulator. *Pask*$^{-/-}$ mice are resistant to high fat diet-induced metabolic disorders. Interestingly, *Pask*$^{-/-}$ mice exhibited almost complete protection from hepatic steatosis, but the mechanism underlying this phenotype was unknown. Here, we show that PASK promotes hepatic lipogenesis by activating SREBP-1c. This regulation occurs at the proteolytic maturation step of SREBP-1c, where the endoplasmic reticulum-bound precursor SREBP-1c undergoes proteolytic

cleavages to liberate the transcriptionally active fragment of the protein. SREBP-1c maturation is strongly induced by feeding and insulin signaling, a condition that also stimulates the hepatic expression of PASK. Using genetic and pharmacological approaches, we demonstrate that PASK is required for SREBP-1c maturation in response to feeding and insulin stimulation. Inhibition of PASK results in decreased expression of the lipogenic SREBP-1c target genes and reduced lipid production in cultured cells and in the mouse and rat liver. Importantly, administration of a PASK inhibitor not only improves hepatic steatosis and whole-body dyslipidemia, but also partially reverses insulin resistance in animal models of diet-induced obesity and dyslipidemia, indicating that PASK is a potential therapeutic target for metabolic diseases. These studies not only further our understanding of the physiological functions of PASK, but also provide new insight into the pathogenesis and treatment of NAFLD and other metabolic disorders.

This dissertation is dedicated to my parents.

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CHAPTER 1

INTRODUCTION

1.1 Per-Arnt-Sim (PAS) Kinase

PAS kinase (PASK) is a protein kinase that is conserved from yeast to human. It was first identified through a sequence homology search against the *Bradyrhizobium japonicum* FixL PAS domain, and was subsequently cloned from HeLa cells (Rutter et al., 2001). All PASK orthologs are composed of an N-terminal PAS domain and a C-terminal serine/threonine kinase domain.

1.1.1 PAS Domain

PAS domains are named after Period (Per), Aryl hydrocarbon nuclear receptor (Arnt) and Single-minded (Sim) proteins in which these domains were first identified (Nambu et al., 1991). The PAS domain is found in a wide variety of proteins across phyla, including transcriptions factors, ion channels and kinases (Sabatini and Lynn, 2015). PAS domains commonly function as sensory domains for extra- and intracellular stimuli, such as oxygen, redox state and light (Zhulin et al., 1997). Despite the divergent primary sequences among these proteins, PAS domains adopt a broadly conserved core structure that is composed of a five-stranded antiparallel β-sheet flanked by several α-helices (Taylor and Zhulin, 1999). Most PAS domains are known to contain a central hydrophobic pocket at the core of their structure, which enable them to bind a variety of small ligands, including ATP, heme and flavins (Kitanishi et al., 2008; Monson et al., 1995; Soshilov and Denison, 2008; Stephenson and Hoch, 2001). Sensory information from PAS domains is then relayed via attached effector domains, which leads to a corresponding physiological output.

The PAS domain of PASK shares a similar structure with other PAS domains, suggesting possible functional conservation as a sensory module (Amezcua et al., 2002). The physiological ligand(s) of the PASK PAS domain remains uncharacterized, but *in vitro* compound screening showed that the PAS domain was able to bind small organic molecules in a selective manner (Amezcua et al., 2002). Moreover, mutations of residues within the ligand-binding pocket that mimic the ligand-bound state of the PAS domain resulted in increased activity of PASK *in vitro*. Compared to the full-length PASK, the kinase domain of PASK alone has much higher kinase activity, which can be suppressed by adding the purified PAS domain *in trans* (Rutter et al., 2001). These results suggest an overall model where the PAS domain could bind and maintain the kinase domain in an inactive state (Figure 1-1). The binding of a ligand to the PAS domain could disrupt this inhibitory interaction, thereby restoring the kinase activity. This unique feature of PASK also raises an interesting hypothesis in which PASK could sense changes in the cellular environment (such as in nutrients, energy level) via binding of metabolites to the PAS domain, and pass on the signal via phosphorylation of its downstream targets, which then leads to appropriate physiological adaptations.

1.1.2 Kinase Domain

PASK belongs to the calcium/calmodulin-dependent protein kinase (CAMK) protein kinase family. The C-terminus of PASK contains a canonical serine/threonine kinase domain (Rutter et al., 2001). It functions as the effector

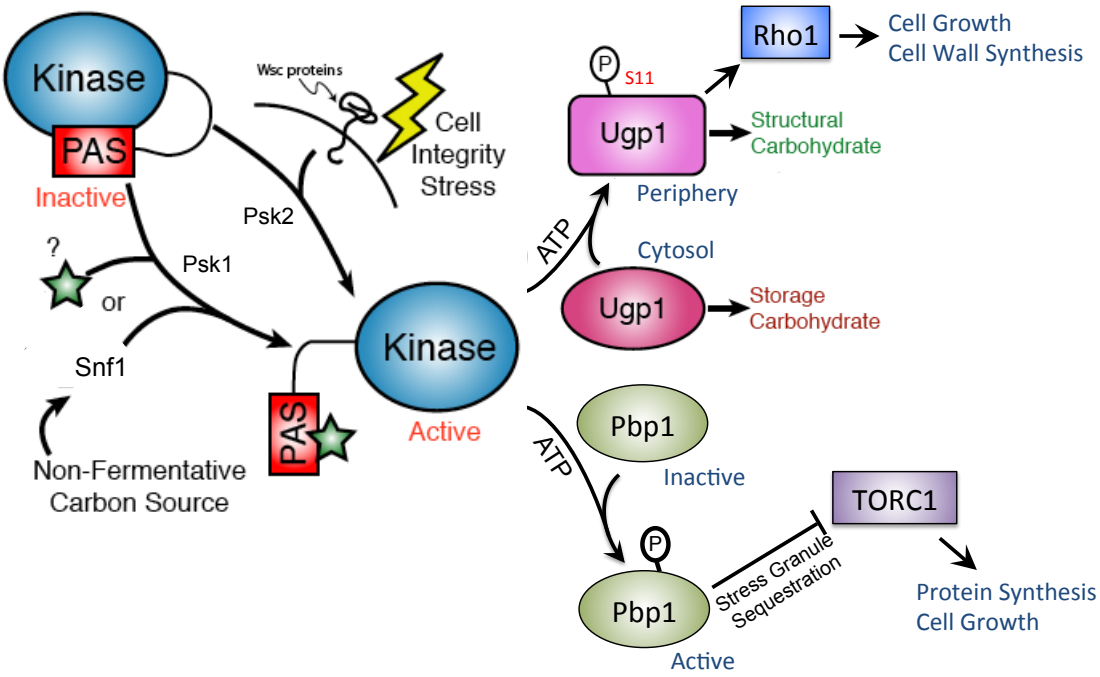

Figure 1-1. PAS kinase function in *S. cerevisiae*. In response to cell integrity stress, Psk2 phosphorylates Ugp1 (p-Ugp1) and triggers its translocation from cytosol to cell periphery, thus causing the redirection of cellular glucose flux from storage towards cell wall synthesis. Psk1 is activated by Snf1 under glucose-depleted conditions (nonfermentative carbon source). Upon activation, Psk1 phosphorylates and activates Pbp1, which inhibits TORC1 via stress granule sequestration.

domain of PASK upon signaling, and most likely mediates its cellular effect via phosphorylation of its substrates (Cardon and Rutter, 2012). Structural and biochemical studies have revealed several unique features of the kinase domain of PASK (Kikani et al., 2010). Most protein kinases require phosphorylation on one or more residues in the activation loop, a conserved loop motif within the kinase domain, to be converted from an inactive to an active form (Johnson et al., 1996). Most PASK orthologs contain a phosphorylatable threonine residue (T1161) within its activation loop. However, phosphorylation at this residue is not required for activation of PASK, as evidenced by no difference in autophosphorylation or substrate phosphorylation between the wild-type and the threonine to alanine (T1161A) mutant form of human PASK. Interestingly, the two *S. cerevisiae* PASK orthologs, which have a valine residue at this position, are catalytically active as well. Although activation loop phosphorylation is dispensable for basal activity of PASK, it is possible that such phosphorylation may become important in regulating PASK activity under certain conditions.

Another unique feature of the PASK kinase domain lies in the substrate specificity. Using peptide library screening, we have determined the consensus phosphorylation sequence for human PASK (H/K/R-X-K/R-X-X-*S/*T, where *S/*T is the target residue, and X is any amino acid) (Kikani et al., 2010). The preference for a histidine residue at the -5 site is unusual among serine/threonine kinases, and has only been observed in two other kinases, human large tumor suppressor kinase (LATS) (Hao et al., 2008) and its yeast homolog Cbk1 (Mazanka et al., 2008). The consensus sequence identified for human PASK

substrates matches the phosphorylation motif of UDP-glucose pyrophosphorylase 1 (Ugp1) (6-HTKTHS*-11), a *bona fide* substrate of PASK in *S. cerevisiae* (Rutter et al., 2002). This suggests that all PASK orthologs share common substrate specificity, which may be instructive in identifying new PASK substrates.

1.1.3 PAS Kinase Function in *S. cerevisiae*

There are two PASK paralogs in *S. cerevisiae*, *PSK1* and *PSK2* (Rutter et al., 2002). They are highly similar in sequence and partially redundant in function. Proteomic, genetic and biochemical studies over the years have provided evidence that PASK regulates multiple cellular processes in response to environmental changes (Figure 1-1).

1.1.3.1 PAS Kinase and Glucose Partitioning

Genetic and biochemical studies in yeast have established a role for PASK in regulating glucose partitioning in response to alterations in the cellular environment. Yeast PASK is activated upon cell integrity stress, which can be induced by heat shock or detergent treatment to the cells (Smith and Rutter, 2007). Alternatively, overexpression of Wsc1, a membrane stress sensory protein, also activates yeast PASK (Grose et al., 2009), potentially by mimicking the stress response. The exact mechanism of how cell integrity stress activates PASK remains unclear. Nonetheless, upon activation, PASK directly phosphorylates Ugp1 at the serine-11 residue within the consensus sequence

described above (Rutter et al., 2002). Ugp1 is an enzyme that converts UTP and glucose-1-phosphate to UDP-glucose, which is the primary glucose donor for the production of glycogen, the major storage form of glucose, and glucan, the major structural constituent of the yeast cell wall (Daran et al., 1995). Interestingly, PASK phosphorylation of Ugp1 does not affect its enzymatic activity, but rather triggers a conformational change of the enzyme and its recruitment to the cell periphery, where cell wall synthesis takes place (Smith and Rutter, 2007). As a result, UDP-glucose generated by phosphorylated Ugp1 is mainly used to produce cell wall glucan. Consistent with this model, PASK null strain (*psk1Δ psk2Δ*) showed hyperaccumulation of glycogen as well as increased sensitivity to cell integrity stress compared to the wild-type strain. Taken together, these results establish a model where, in response to cell integrity stress, PASK directs glucose flux towards glucan synthesis at the expense of glycogen storage, which promotes cell wall stabilization and cell survival.

Glucose is the preferred carbon source for yeast, which they can ferment into ethanol. When the glucose level decreases, yeast must switch to utilize other carbon sources for continuous growth. In addition to cell integrity stress, yeast PASK is also activated by nonfermentative carbon sources (Grose et al., 2009). This activation is dependent on Snf1, the yeast homolog of mammalian AMPK. Snf1 is activated under glucose-depleted conditions, which then activates PASK, potentially by direct phosphorylation (discussed below) (DeMille et al., 2015). Activation of PASK under this condition also leads to Ugp1 phosphorylation (Grose et al., 2009). Therefore, it is possible that when glucose is limited, PASK

switches the utilization of the available glucose from storage to other more important cellular processes.

1.1.3.2 PAS Kinase and Cell Growth

In addition to its role in glucose partitioning, phosphorylation of Ugp1 by PASK is also involved in regulating cell growth. Yeast strains harboring a temperature-sensitive allele of Target of Rapamycin 2 (*tor2*^{ts}) fail to grow at the restrictive temperature (37 °C) (Helliwell et al., 1994). This phenotype, however, can be suppressed by overexpression of yeast PASK (Cardon et al., 2012), or by treatment of cell wall perturbing agents (Bickle et al., 1998), which activate PASK. Interestingly, the rescue of *tor2*^{ts} mutant requires Ugp1 phosphorylation as well as activation of Rho1, a small GTPase and a downstream effector of Tor2 in mediating temporal growth. Further studies showed that phosphorylated Ugp1 nucleates the formation of a signaling complex at the cell membrane that includes Rom2, a Rho1 activating guanine nucleotide exchange factor, and Ssd1, a RNA binding protein. This complex activates Rho1 GTPase, which in turn stimulates the mitogen activated protein kinase (MAPK) signaling pathway and promotes cell growth and division (Helliwell et al., 1998). These observations suggest that yeast PASK can coordinate cell growth by phosphorylating Ugp1, which initiates a progrowth signaling cascade as well as increases the production of precursors for cell wall biogenesis.

PASK may also control cell growth by regulating protein synthesis. A yeast proteomic screen for PASK substrates has identified several proteins that are

involved in protein translation, such as Cap-associated factor 20 (Caf20), the yeast homolog of mammalian 4E-BP, Tif11, the yeast eukaryotic translation initiation factor 1A (eIF1A), and Sro9, an mRNA-binding protein (Rutter et al., 2002). All three proteins can be phosphorylated by PASK *in vitro*. However, the physiological relevance of these phosphorylation events awaits further investigation. In yeast, protein translation is strongly regulated by target of rapamycin complex 1 (TORC1) (Loewith et al., 2002). Interestingly, a recent study suggests that PASK may act as a negative regulator of TORC1 (DeMille et al., 2015). Under nutrient depleted conditions, Snf1 phosphorylates and activates PASK, which in turn leads to phosphorylation and activation of poly(A)-binding protein binding protein 1 (Pbp1), a component of stress granules. Active Pbp1 then inhibits TOC1 by sequestering the complex at stress granules.

1.1.4 PAS Kinase Function in Mammals

The specific glucose partitioning function of PASK in yeast described above is not conserved in mammals. PASK phosphorylation site in yeast Ugp1 is not conserved in its human or mouse homolog, nor is human UGP1 phosphorylated by human PASK *in vitro* (Hao and Rutter, 2008). Although differing in mechanism, mammalian PASK has been shown to participate in coordinating cellular and organismal metabolism with environmental changes (Figure 1-2).

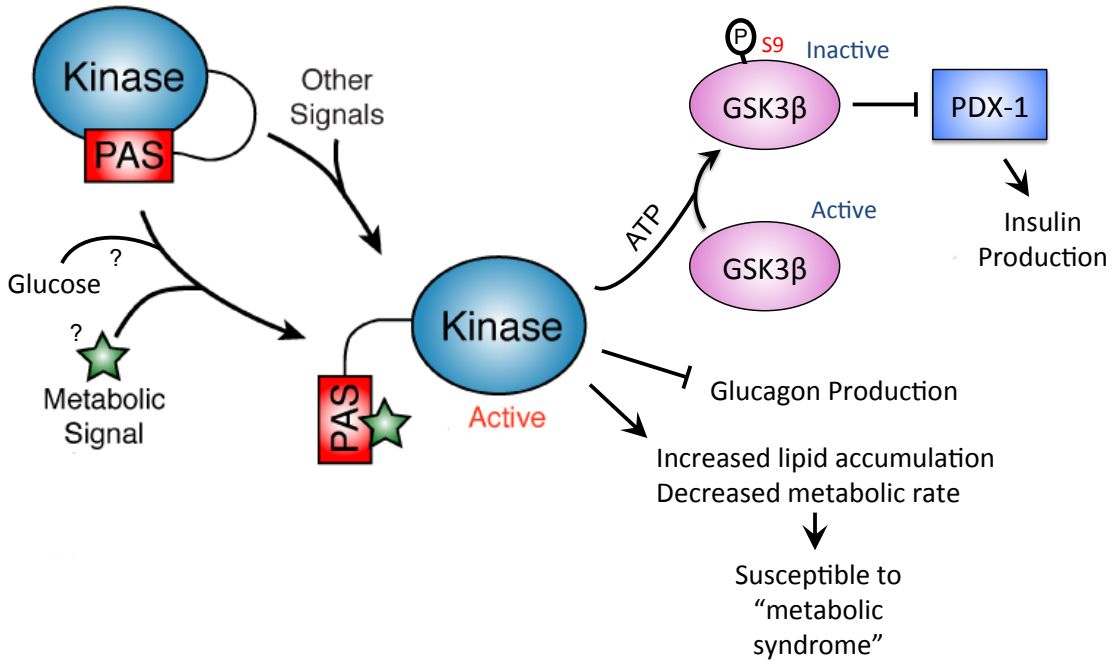

Figure 1-2. PAS kinase function in mammals. In pancreatic β-cells, PASK stimulates the expression of the insulin gene via PDX-1 transcription factor. PASK phosphorylates and inhibits GSK3β, thus promoting the protein stability of PDX-1. PASK also suppresses glucagon production in α-cells and regulates lipid metabolism in liver. Upstream signaling cues that mediate PASK activation remain largely elusive. Glucose stimulation in pancreatic β-cells has been shown to stimulate PASK expression, as well as its activity.

1.1.4.1 PAS Kinase and Insulin Production

Studies using Min6 cells, a mouse pancreatic β-cell line, and isolated rat islets indicate that PASK plays a role in regulating insulin production. Incubation of cells with high glucose not only increased the expression of PASK, but also promoted its kinase activity (da Silva Xavier et al., 2004). Overexpression of wild-type PASK stimulated, while kinase-inactive PASK inhibited, the transcription of the preproinsulin gene (hereafter referred to as the insulin gene) under basal and high glucose conditions, respectively. Inhibition of PASK, either by RNAi silencing or by microinjection of PASK antibody, also abolished glucose-stimulated transcription of the insulin gene. The effect of PASK on the insulin gene promoter is mediated by pancreatic duodenum homeobox-1 (PDX-1) transcription factor. PASK promotes the transcription of *PDX-1* gene, as silencing of PASK or overexpression of kinase-inactive PASK decreased glucose-induced *PDX-1* gene expression. In addition, PASK also promotes the stability of PDX-1 protein (Semache et al., 2013). Under low glucose condition, PDX-1 is phosphorylated by glycogen synthase kinase 3β (GSK3β), which triggers its proteasomal degradation (Humphrey et al., 2010). This process is inhibited under high glucose condition. A recent study showed that overexpression of wild-type PASK mimicked the effect of glucose stimulation on PDX-1 protein stability. PASK inhibited GSK3β by direct phosphorylation at serine-9 position, which in turn lowered PDX-1 phosphorylation level and prevented its degradation (Semache et al., 2013). Again, this effect was reversed by PASK silencing or overexpression of kinase-inactive PASK in Min6 cells or isolated rat islets.

Two SNPs, L1051V and G1117E, have been identified in the human PASK gene that are associated with maturity-onset diabetes of the young (MODY), a dominantly inherited form of early onset diabetes (Semplici et al., 2011). The G1117E mutation increased PASK activity *in vitro* and enhanced basal insulin secretion when expressed in isolated mouse islets. These observations further support a positive role of PASK in regulating insulin production.

1.1.4.2 PAS Kinase and Glucagon Production

Another function of PASK in the pancreas is to regulate glucagon production (da Silva Xavier et al., 2011). The expression level of PASK is approximately two-fold higher in α-cells compared to that in β-cells. Whole-body PASK knockout (*Pask*$^{-/-}$) mice showed increased fasting blood glucose and plasma glucagon level compared to wild-type mice. Consistent with that, islets isolated from *Pask*$^{-/-}$ mice secreted more glucagon and less insulin than in wild-type islets. Further studies showed that *PASK* silencing in α-TC1-9 cells blocked glucose's ability to suppress glucagon secretion, while PASK overexpression in the same cells, as well as in human islets, inhibited glucagon secretion. The regulation of glucagon production by PASK likely occurs at the level of transcription, and is potentially mediated by AMPK-α2. However, the detailed mechanism still requires further investigation. Interestingly, the same study showed that PASK expression was significantly decreased in islets from human type 2 diabetes (T2D) patients compared to healthy controls. This downregulation

of PASK may potentially lead to increased fasting and postprandial glucagon level, as well as decreased postprandial insulin level; all of which are associated with T2D and other metabolic diseases.

1.1.4.3 Characterization of PAS Kinase Knockout Mice

To further study the physiological function of mammalian PASK, our lab studied germline PASK knockout (*Pask*$^{-/-}$) mice (Hao et al., 2007). *Pask*$^{-/-}$ mice are viable and fertile with no obvious developmental defects. Consistent with the results from cultured Min6 cells and islets, *Pask*$^{-/-}$ mice showed impaired insulin secretion in response to glucose stimulation. No changes in islet morphology or β-cell mass were observed upon PASK deficiency. When fed with a standard normal chow diet, *Pask*$^{-/-}$ mice exhibited no difference in metabolism compared to their wild-type littermates. Interestingly, when challenged with a high-fat diet (HFD), *Pask*$^{-/-}$ mice showed decreased susceptibility to diet-induced metabolic disorders. Unlike their wild-type littermates, PASK deficient mice gained less weight, showed improved insulin sensitivity and elevated metabolic rate. The most profound phenotype observed in these mice, however, was the nearly complete protection from HFD-induced triglyceride accumulation in liver (hepatic steatosis). This is also accompanied by decreased expression of genes involved in lipid metabolism, such as fatty acid transporter (*Cd36*), stearoyl-CoA desaturase 1 (*Scd1*) and peroxisome proliferator-activated receptor γ (*Pparγ*). These data suggest a potential role of PASK in regulating lipid metabolism in liver, and here, we show that PASK activates fatty acid and triglyceride synthesis

through sterol regulatory element binding protein 1 (SREBP-1), the master transcription factor that drives the lipogenic program in liver.

1.2 Sterol Regulatory Element Binding Proteins (SREBPs)

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that belong to the basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family (Brown and Goldstein, 1997). This family was first identified in 1993 by Michael Brown and Joseph Goldstein's group as transcription factors for genes involved in lipid metabolism (Briggs et al., 1993; Wang et al., 1993). In mammals, there are three isoforms in this family. SREBP-1a and SREBP-1c are produced from a single gene *SREBF-1* using alternative promoters (Hua et al., 1995b). These two isoforms only differ in their respective first exons. SREBP-2 is encoded by a separate gene *SREBF-2*. SREBP-1a and SREBP-2 are ubiquitously expressed at a relatively constant level, while SREBP-1c is the predominant isoform in metabolic tissues including liver and white adipose tissue (Shimomura et al., 1997; Ye and DeBose-Boyd, 2011).

1.2.1 SREBP Domain Structure

The domain organization of SREBPs reveals a unique feature of this transcription factor family. All SREBPs possess (1) a transcriptional activation domain (TAD) located at the extreme N-terminus of the protein, followed by a bHLH-LZ domain that mediates DNA binding and protein dimerization; (2) a middle segment that contains two membrane-spanning helices separated by a

short hydrophilic sequence; and (3) a C-terminal regulatory domain (Brown and Goldstein, 1997). The full-length SREBPs are membrane-bound proteins and require a proteolytic activation step, known as SREBP maturation, to release the transcriptionally active N-terminal portion of the transcriptions factors.

1.2.2 SREBP Maturation

All SREBPs are first synthesized as inactive precursor forms that are anchored on the endoplasmic reticulum (ER) membrane by the two transmembrane domains in a hairpin configuration: the N-terminal and C-terminal domains face the cytoplasmic side of the ER membrane, while the hydrophilic loop between the transmembrane domains projects into the ER lumen (Figure 1-3) (Brown and Goldstein, 1997; Hua et al., 1995a). In order for the N-terminal transcriptionally active portion of SREBPs to enter the nucleus, the precursor SREBPs undergo a maturation process, during which the precursor forms translocate from the ER to the Golgi membrane where two proteolytic cleavage events release the N-terminal fragment of SREBPs (Brown and Goldstein, 2009; Ye and DeBose-Boyd, 2011). A number of ER and Golgi membrane proteins, including SCAP, Insig, S1P and S2P play critical roles in regulating and performing SREBP maturation.

1.2.2.1 SREBP Cleavage Activating Protein (SCAP)

SREBP cleavage activating protein (SCAP) is a polytopic ER membrane protein (Hua et al., 1996; Nohturfft et al., 1998; Sakai et al., 1997). The N-

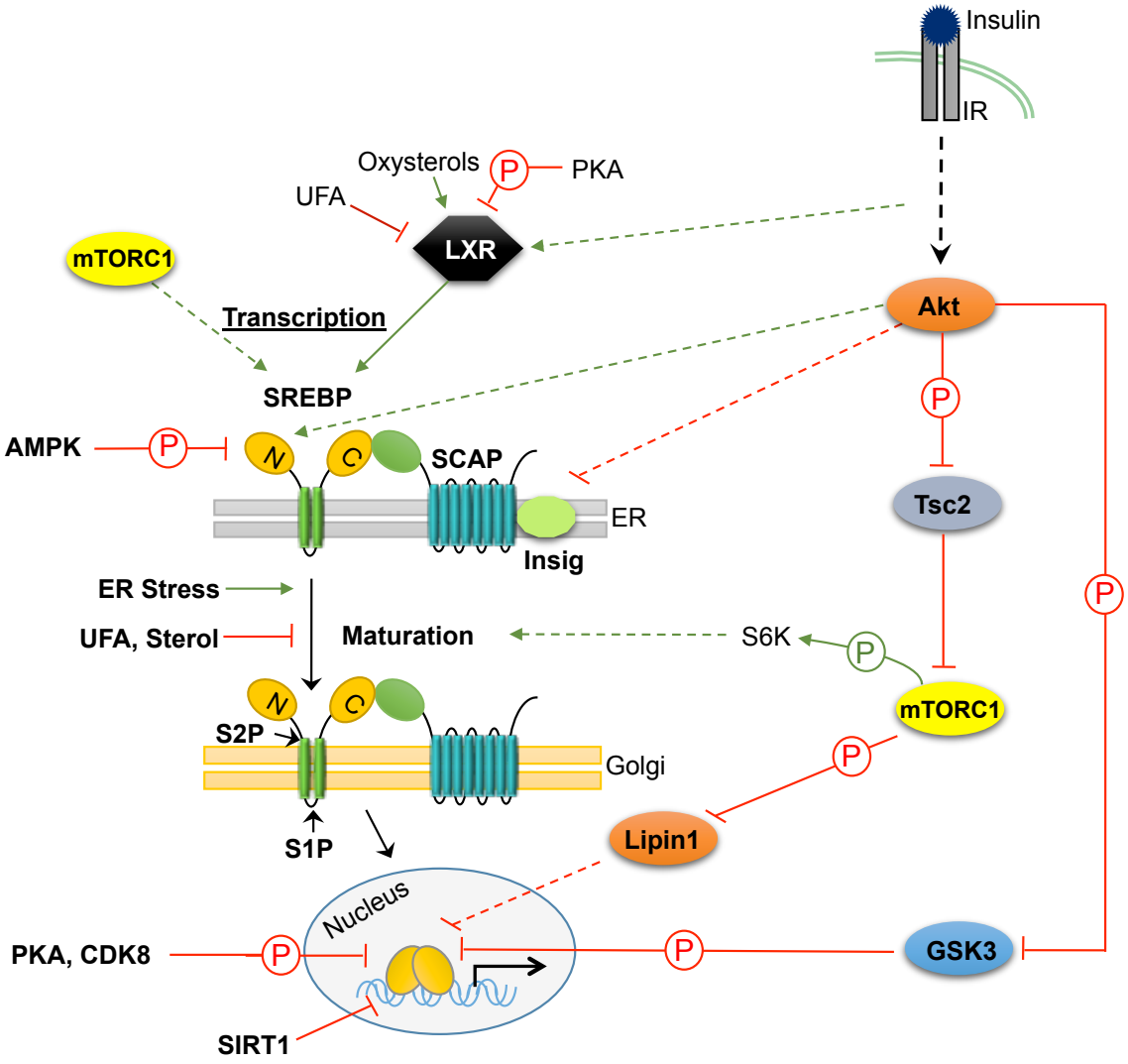

Figure 1-3. Regulation of SREBP activation. SREBPs are highly regulated at multiple levels in response to a wide range of intrinsic and extrinsic signals. Regulation occurs at the levels of transcription, posttranslational proteolytic maturation, transcriptional activity and also protein stability. Positive regulation is shown in green, whereas negative regulation is shown in red. Ⓟ indicates phosphorylation. See text for details.

terminal part of SCAP contains eight transmembrane helices, and the C-terminal cytosolic part contains a tryptophan-aspartate-repeat (WD) domain that interacts with the C-terminal regulatory domains of SREBPs. SCAP escorts the translocation of SREBPs from the ER to the Golgi by interacting with the Sec23/24 subunit of the coat protein complex II (COPII) trafficking machinery, thus allowing the SCAP/SREBP complex to be exported from the ER via COPII-coated vesicles (Espenshade et al., 2002; Nohturfft et al., 2000). The interaction between SCAP and the Sec23/24 subunit is mediated through the core sequence MELADL within the cytosolic loop 6 (between transmembrane helices 6 and 7) of the SCAP protein. Once SREBPs undergo proteolysis on the Golgi membrane, SCAP is released and recycled back to the ER (Sun et al., 2005; Sun et al., 2007). SCAP is required for SREBP maturation (Sakai et al., 1998a). Liver-specific disruption of the *Scap* gene significantly lowered the levels of the mature SREBPs, decreased expression of their lipogenic target genes and also reduced lipid synthesis in liver (Matsuda et al., 2001; Moon et al., 2012).

1.2.2.2 Insulin-Induced Genes (Insigs)

Insulin induced genes (Insigs) are a family of ER membrane resident proteins (Dong and Tang, 2010). There are two isoforms in mammalian cells, designated as Insig1 and Insig2. Insig1 was first identified in regenerating rat liver (Peng et al., 1997), and its expression is induced by insulin treatment (Diamond et al., 1993). Rodent *Insig2* gene encodes two transcript variants, *Insig2a* and *Insig2b*, using alternative promoters with noncoding first exons (Yabe et al., 2002;

Yabe et al., 2003). *Insig2b* is constitutively expressed in most tissues, while *Insig2a* is the predominant form in liver (Yabe et al., 2003). Although the 5' untranslated region (5' UTR) is different between *Insig2a* and *Insig2b*, they both are translated into the same protein product, Insig2. In human, INSIG1 (277 amino acids) and INSIG2 (225 amino acids) proteins are 59% identical in amino acid sequence (Yabe et al., 2002; Yang et al., 2002). Both isoforms contain six transmembrane helices (Feramisco et al., 2004; Goldstein et al., 2006). Insig proteins negatively regulate SREBP maturation by interacting with SCAP and retaining the SCAP/SREBP complex on the ER membrane (Adams et al., 2004; Yang et al., 2002). Deletion of both Insig isoforms in mouse liver resulted in accumulation of mature SREBPs, increased expression of their lipogenic target genes and elevated rate of lipid synthesis (Engelking et al., 2005). Overexpression of human INSIG1 in mouse liver, on the other hand, caused the opposite effects on SREBP maturation and lipogenesis (Engelking et al., 2004).

1.2.2.3 Site-1 Protease (S1P) and Site-2 Protease (S2P)

Once transported to the Golgi, SREBPs undergo two sequential proteolytic cleavage events to liberate the N-terminal part of the protein (Sakai et al., 1996). The first cleavage occurs at a conserved leucine residue (RXXL/) within the luminal loop between the two transmembrane helices (Duncan et al., 1997; Sakai et al., 1998b). This step divides SREBPs into two halves, each still embedded in the membrane by a single transmembrane helix. This first proteolytic cleavage is mediated by site-1 protease (S1P), a membrane-bound serine protease with a

luminal catalytic domain (Rawson et al., 1998; Sakai et al., 1998b). The second cleavage occurs within the first transmembrane helix of SREBPs, which liberates the N-terminal fragment of SREBPs (Duncan et al., 1998). This proteolysis step is mediated by site-2 protease (S2P), a membrane-bound hydrophobic zinc metalloprotease whose active site (HEXXH) is embedded within the membrane (Rawson et al., 1997; Zelenski et al., 1999). S1P or S2P deficient cells are auxotrophic for cholesterol, indicating their essential roles in generating transcriptionally active form of SREBPs (Rawson et al., 1998; Sakai et al., 1996). Indeed, liver-specific disruption of the *S1p* gene led to significant decrease in mature SREPBs levels, lipogenic gene expression, as well as lipid production (Yang et al., 2001).

The N-terminal fragments of SREBPs, also known as the mature forms, contain a nuclear localization signal that facilitates their translocation into the nucleus to activate the transcription of their target genes (Nagoshi and Yoneda, 2001).

1.2.3 Transcription Activation by SREBPs

After proteolytic maturation, the mature forms of SREBPs drive transcription of their target genes by binding to specific sequence motifs within the promoters of their targets. Unlike other bHLH-LZ family transcription factors, which typically bind to a palindromic E-box DNA motif (5'-CANNTG-3') (Murre et al., 1994), the mature SREBPs can also bind to a 10-base-pair (bp) motif called sterol regulatory element (SRE) (5'-ATCACCCCAC-3') (Kim et al., 1995; Smith et

al., 1990). This is because SREBPs have a tyrosine residue in their DNA binding basic domains instead of an arginine that is found in all other bHLH-LZ transcription factors (Kim et al., 1995). Structural studies suggest that the tyrosine residue causes a conformational change in the basic domains, which allows SREBPs to bind to the SRE with high affinity (Parraga et al., 1998).

In addition to recognizing SREs in the promoter regions of the target genes, the mature forms of SREBPs also recruit non-DNA-binding transcription coactivators through their N-terminal TADs to further facilitate transcriptional activation (Osborne and Espenshade, 2009; Sato et al., 1994). Both SREBP-1a and SREBP-2 have longer TADs that contain conserved residues required for their interaction with transcription coactivators, such as CBP/p300, a histone acetyltransferase, and Mediator, a multi-subunit complex essential for RNA polymerase II mediated transcription (Oliner et al., 1996; Toth et al., 2004; Yang et al., 2006). These residues are not present in the shorter SREBP-1c TAD due to the alternative first exon (Toth et al., 2004). As a result, SREBP-1c interacts with CBP/p300 and Mediator with much lower affinity, thus attenuating its transactivation potential.

1.2.4 Function of SREBPs

All SREBPs regulate lipid synthesis with distinct and overlapping functions. SREBP-1c primarily stimulates the transcription of genes involved in fatty acid and triglyceride biosynthesis, while SREBP-2 mainly drives the transcription of genes involved in cholesterol biosynthesis (Horton et al., 2002; Pai et al., 1998).

SREBP-1a can activate genes in both biosynthesis pathways. Various mouse models have been made to manipulate SREBPs levels. Germline *SREBP-1* knockout mice, which lack both SREBP-1a and SREBP-1c, are partially embryonic lethal (Shimano et al., 1997b). The surviving knockout mice showed increased expression of SREBP-2 and its target genes, as well as elevated cholesterol synthesis in liver. Germline *SREBP-2* knockout mice, on the other hand, are completely embryonic lethal. These results indicate that SREBP-2 can compensate for the loss of SREBP-1, while *SREBP-2* itself is an essential gene. Disruption of *SREBP-1c* alone does not lead to embryonic lethality (Liang et al., 2002). Mice lacking SREBP-1c showed decreased hepatic expression of enzymes involved in fatty acid biosynthesis, reduced fatty acid synthesis and increased cholesterol synthesis caused by SREBP-2 compensation.

Transgenic mice have also been generated to specifically express the mature form of each SREBP isoform in the liver. Hepatic overexpression of mature SREBP-1a greatly promoted the expression of genes involved in the fatty acid, triglyceride and cholesterol biosynthesis pathways (Shimano et al., 1996). This was accompanied by a 20-fold increase in fatty acid and triglyceride synthesis and a five-fold increase in cholesterol synthesis. Overexpression of mature SREBP-1c in liver, on the other hand, only induced triglyceride accumulation by five-fold (Shimano et al., 1997a), consistent with it being a weaker transcription factor. Interestingly, no changes in cholesterol level were observed in these animals. In contrast to SREBP-1, hepatic overexpression of SREBP-2 favored cholesterol biosynthesis, resulting in a 20-fold increase in

cholesterol level and a four-fold increase in triglyceride level in liver (Horton et al., 1998).

Recent studies have shown that SREBPs target genes are not limited to lipogenic enzymes or confined to liver. For example, in macrophages, SREBP-1a facilitates the pro-inflammatory response by activating the expression of Nlrp1a, a subunit of the inflammasome that mediates the activation of pro-inflammatory cytokines (Im et al., 2011). Additionally, a genome-wide ChIP-sequencing study revealed that SREBP-2 occupies the promoter regions of genes involved in autophagy, implicating the involvement of SREBP-2 in the regulation of genes governing autophagy (Seo et al., 2011).

1.2.5 Regulation of SREBPs

As the master transcriptional regulators of lipogenesis in mammalian cells, SREBPs are tightly regulated at multiple levels in response to a variety of extracellular and intracellular signals. This allows cells to coordinate their metabolic status with changes in the cellular environment. Here, we discuss the effects of various signals on SREBP gene transcription, proteolytic maturation, transcriptional activity and protein stability (Figure 1-3).

1.2.5.1 Regulation of SREBPs by Sterol

Studies have demonstrated that maturation of SREBP-1a and SREBP-2 is subjected to feedback inhibition by sterol (Figure 1-3). Under sterol-depleted conditions, the SCAP/SREBP complex migrates to the Golgi via COPII-coated

vesicles (Espenshade et al., 2002; Nohturfft et al., 2000; Sun et al., 2005). Mature SREBPs are released from the Golgi membrane and in turn enter the nucleus to activate genes involved in cholesterol biosynthesis. When intracellular sterol levels are restored, cholesterol directly binds to the sterol-sensing domain of SCAP (transmembrane helices 2-5) (Radhakrishnan et al., 2004), which triggers a conformational change in the cytosolic loop 6 that contains the binding site for the Sec23/24 subunit of the COPII trafficking machinery (Brown et al., 2002; Sun et al., 2005). This conformational change triggers the association of SCAP with Insig proteins, which disrupts its interaction with the Sec23/24 subunit. Cholesterol derivatives oxysterols also promote SCAP-Insig interaction by directly binding to Insig proteins (Radhakrishnan et al., 2007). As a result, the SCAP/SREBP complex is retained in the ER, which leads to a decreased rate of cholesterol synthesis.

Insig1 also plays a role in the feedback inhibition of SREBP maturation. Upon sterol depletion, Insig1 dissociates from SCAP and becomes susceptible to ubiquitination and proteasomal degradation (Gong et al., 2006). This process is mediated by glycoprotein 78 (gp78), a membrane-bound E3 ubiquitin ligase (Lee et al., 2006b). Under sterol-repleted conditions, SCAP binds to Insig1, which precludes its interaction with gp78, thus leading to Insig1 stabilization. Interestingly, *Insig1* itself is an SREBP target gene (Yabe et al., 2002). Therefore, activation of SREBPs not only elevates sterol level, but also promotes Insig1 protein accumulation on the ER membrane, both of which inhibit further activation of SREBPs in a convergent manner. Unlike Insig1, Insig2 protein has a much

lower turnover rate that is not affected by sterol (Lee et al., 2006a). *Insig2* expression is not activated by SREBPs, either (Yabe et al., 2002). The role of Insig2 in regulating SREBP maturation will be discussed below.

In addition to SREBP maturation, oxysterols can also activate *SREBP-1c* transcription and fatty acid synthesis. Oxysterols serve as agonists of nuclear receptor liver X receptor (LXR) and promote its binding to the *SREBP-1c* promoter (DeBose-Boyd et al., 2001; Repa et al., 2000; Yoshikawa et al., 2001). It is proposed that under sterol-replete conditions, cells increase fatty acid synthesis to promote cholesterol esterification.

1.2.5.2 Regulation of SREBPs by Insulin Signaling

Insulin is a hormone that is secreted by pancreatic β-cells in response to elevated levels of blood glucose and other nutrients following food intake (Samuel and Shulman, 2012). It acts on metabolic tissues to promote nutrient absorption and storage for future use. In muscle, insulin stimulates glucose uptake and glycogen synthesis. In white adipose tissue, insulin also promotes glucose uptake and lipid synthesis while suppressing lipolysis. In liver, insulin inhibits glucose production via gluconeogenesis and at the same time activates glycogen synthesis and lipogenesis. All these actions are carried out by an intricate network of signaling events initiated by insulin.

1.2.5.2.1 Insulin Signaling Pathway in the Liver

The insulin signaling pathway in liver is initiated by binding of insulin to the insulin receptor (IR) tyrosine kinase, which triggers its dimerization and auto-phosphorylation (Saltiel and Kahn, 2001). Activated IR recruits and phosphorylates insulin receptor substrates 1/2 (IRS1/2) on tyrosine residues, which then serve as binding sites for phosphatidylinositol-3-OH kinase (PI3K), leading to its activation. PI3K is a lipid kinase that phosphorylates phosphoinositide-4,5-phosphate (PIP2) at 3'-OH position to generate phosphoinositide-3,4,5-phosphate (PIP3) on the plasma membrane. PIP3 is then recognized by the pleckstrin homology (PH) domains of 3-phosphoinositide dependent kinase 1 (PDK1) and protein kinase B (PKB)/Akt, which leads to the membrane translocation of these two kinases and the subsequent phosphorylation of Akt by PDK1 at threonine residue 308. In addition to PDK1 phosphorylation, Akt is also phosphorylated by the mechanistic target of rapamycin (mTOR) complex 2 (mTORC2) at serine residue 473 (Vadlakonda et al., 2013). Both phosphorylation events are required for the full activation of Akt. Once activated, Akt phosphorylates a wide range of downstream targets to modulate cell metabolism and other functions.

1.2.5.2.1.1 Insulin and hepatic glucose metabolism. Insulin regulates hepatic glucose metabolism through two mechanisms, both mediated by Akt (Saltiel and Kahn, 2001). First, insulin inhibits gluconeogenesis to prevent further elevation of blood glucose concentration. Akt phosphorylates the transcription factor Forkhead box protein 1 (FoxO1), resulting in its nuclear exclusion and the

downregulation of its gluconeogenic target genes. Second, insulin stimulates glycogen synthesis to promote glucose storage. Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3), which in turn leads to the activation of glycogen synthase (GS) and increased glycogen synthesis.

1.2.5.2.1.2 Insulin and hepatic lipid metabolism. Insulin promotes the conversion of dietary carbohydrates (*de novo* lipogenesis) and fatty acids (fatty acid esterification) into triglyceride in liver, which is then transported to white adipose tissue for long-term storage (Krycer et al., 2010; Saltiel and Kahn, 2001). SREBP-1c is the major transcriptional regulator of fatty acid and triglyceride synthesis in liver in response to insulin signaling. Growing evidence indicates that insulin activates SREBPs at multiple levels, including gene transcription, proteolytic maturation, transcriptional activity and protein stability.

1.2.5.2.2 Effect of Insulin Signaling on SREBP Transcription

Insulin selectively regulates the transcription of *SREBP-1c* (Osborne and Espenshade, 2009; Zhang et al., 2005). The mRNA level of *SREBP-1c* in mouse and rat liver was reduced under fasted conditions and was robustly induced upon feeding, which stimulates the release of insulin (Horton et al., 1998; Li et al., 2010). Rats treated with streptozotocin, a chemical that causes pancreatic β-cell failure and insulin deficiency, showed a profound decrease in hepatic *SREBP-1c* mRNA level (Shimomura et al., 1999b). This decrease was completely reversed by administration of insulin. The effect of insulin on *SREBP-1c* transcription can also be recapitulated in isolated rat primary hepatocytes (Chen et al., 2004; Li et

al., 2010). These results indicate that insulin is a potent activator of *SREBP-1c* transcription.

Early studies demonstrated that the LXR nuclear receptor is required for *SREBP-1c* transcriptional activation by insulin (Figure 1-3). Mutating the LXR binding elements (LXRE) in the *SREBP-1c* promoter abolished its ability to respond to insulin stimulation (Chen et al., 2004). The mechanism of how insulin activates LXR remains unclear. In addition, SREBP-1c can also activate its own transcription by binding to the SRE motif in its own promoter (Amemiya-Kudo et al., 2000), thus establishing a feed-forward transcriptional regulatory loop upon insulin stimulation.

Recent studies have shown that insulin-stimulated *SREBP-1c* transcription depends on PI3K, Akt and mTOR complex 1 (mTORC1) (Figure 1-3) (Li et al., 2010). mTORC1 is a major downstream effector of Akt (Sarbassov et al., 2005). Upon insulin stimulation, Akt directly phosphorylates and inactivates tuberous sclerosis 2 (TSC2) within the TSC1-TSC2 complex. TSC2 is a GTPase activating protein (GAP) for Ras homolog enriched in brain (Rheb) small GTPase. Phosphorylation of TSC2 by Akt causes an increase in GTP-bound Rheb, which in turn leads to mTORC1 activation. In rat primary hepatocytes, insulin-induced transcriptional activation of *SREBP-1c* was blocked by small molecule inhibitors of PI3K (wortmannin), Akt (Akti-1/2) and mTORC1 (rapamycin) (Li et al., 2010), indicating that mTORC1 plays an essential role in activating *SREBP-1c* transcription upon insulin stimulation. Interestingly, pharmacological inhibition of p70 ribosomal S6 kinase (S6K), a kinase directly activated by mTORC1, had no

effect on *SREBP-1c* transcription, suggesting an alternative downstream effector of mTORC1 in this regulatory process.

1.2.5.2.3 Effect of Insulin Signaling on SREBP Maturation

In addition to transcription, insulin also stimulates SREBP-1c maturation. In rat primary hepatocytes, insulin treatment rapidly increased the abundance of mature SREBP-1 without affecting the levels of *SREBP-1* mRNA or the precursor form (Hegarty et al., 2005). A similar effect was also observed in rat liver with constitutive expression of human SREBP-1c (Owen et al., 2012), indicating that insulin can specifically activate SREBP-1c maturation.

Similar to transcriptional activation, proteolytic activation of SREBP-1c by insulin also requires PI3K, Akt and mTORC1 (Figure 1-3). Pharmacological inhibition and siRNA knockdown of these three components in the insulin signaling pathway both led to decreased SREBP-1c maturation (Owen et al., 2012; Porstmann et al., 2008), indicating their essential roles in regulating this process. Whether S6K regulates SREBP-1 maturation downstream of mTORC1 is still under debate. An S6K inhibitor decreased SREBP-1c maturation in rat primary hepatocytes upon insulin stimulation (Owen et al., 2012). A similar effect was observed in TSC1 null mouse embryonic fibroblasts (MEFs), where siRNA knockdown of *S6K* suppressed SREBP-1 maturation induced by mTORC1 hyperactivation (Duvel et al., 2010). However, in a separate study, *S6K* silencing showed no effect on SREBP-1 maturation following Akt activation in wild-type MEFs or in human retinal pigment epithelial cells (Lewis et al., 2011). These

results raise the possibility of an alternative downstream effector of mTORC1 in mediating SREBP-1 maturation.

Insulin also activates SREBP-1 maturation by suppressing *Insig2a* (Figure 1-3) (Yabe et al., 2003). *Insig2a* is the major Insig isoform in liver (Yabe et al., 2003), and Insig2 protein preferentially interacts with the SCAP-SREBP-1c complex (Yellaturu et al., 2009b), causing its ER retention. Upon insulin stimulation, Akt promotes the degradation of *Insig2a* mRNA via its 3' UTR (Yellaturu et al., 2009b), but the detailed mechanism remains unknown. Suppression of *Insig2a* mRNA levels by insulin is not mediated by mTORC1, as rapamycin has no effect on the *Insig2a* mRNA level (Yecies et al., 2011). Therefore, insulin regulates SREBP-1c maturation via two distinct pathways downstream of Akt: one that depends on mTORC1 activation and one that involves *Insig2a* degradation. Both pathways are required for activation of SREBP-1c maturation, as demonstrated by the observations from Tsc1 liver-specific knockout mice (Yecies et al., 2011). Despite hyperactivation of mTORC1 in liver, these mice showed a significant decrease in SREBP-1c target gene expression and failed to develop hepatic steatosis when fed with a HFD. Further studies demonstrated that constitutive activation of mTORC1 resulted in feedback inhibition of insulin/PI3K/Akt signaling, which in turn led to accumulation of *Insig2a* and suppression of SREBP-1c maturation.

It has been reported that insulin can also stimulate SREBP-1c maturation by promoting its phosphorylation (Figure 1-3). In rat primary hepatocytes, insulin treatment led to increased serine and threonine phosphorylation of SREBP-1c in

an Akt-dependent manner, which increased the affinity of SCAP-SREBP-1c complex for the Sec23/24 subunit of the COPII vesicles (Yellaturu et al., 2009a). The kinase that mediates this phosphorylation event, as well as the connection between SREBP-1c phosphorylation and mTORC1 activation, awaits further investigation.

1.2.5.2.4 Effect of Insulin Signaling on Mature SREBPs

The mature forms of SREBPs are highly unstable and are susceptible to ubiquitination and proteasomal degradation (Hirano et al., 2001; Wang et al., 1994). This process depends on the transcriptional activity of SREBPs, as mutations in the TAD or DNA-binding domains of SREBPs stabilize the proteins (Sundqvist and Ericsson, 2003). Upon binding to DNA, SREBP-1a/c recruits GSK3, which phosphorylates SREBP-1a at threonine residue 426 (threonine 402 in SREBP-1c) and serine residue 430 (serine residue 406 in SREBP-1c) (Figure 1-3) (Kim et al., 2004; Punga et al., 2006; Sundqvist et al., 2005). Phosphorylation on these two residues creates a docking site for F-box and WD repeat domain-containing 7 (Fbw7) E3 ubiquitin ligase, which in turn promotes ubiquitination and proteasomal degradation of SREBP-1 (Sundqvist et al., 2005). Upon insulin stimulation, Akt phosphorylates GSK3 at serine residue 9 and inhibits its activity (Cross et al., 1995). As a result, Fbw7 can no longer recognize SREBP-1, thus preventing SREBP-1 from degradation.

A recent study revealed that, in addition to transcriptional and proteolytic activation of SREBPs, mTORC1 also promotes the nuclear abundance of mature

SREBPs. This effect is mediated by lipin 1, a phosphatidic acid phosphatase (Figure 1-3) (Peterson et al., 2011). mTORC1 directly phosphorylates lipin 1 at multiple sites. This phosphorylation prevents its entry into nucleus, which in turn leads to accumulation of nuclear SREBPs. Genetic and pharmacological inhibition of mTORC1 causes lipin 1 nuclear translocation, decreased SREBP nuclear abundance and redistribution of SREBPs to the periphery of nucleus. The same results were also observed using a mutant form of lipin 1 that lacks all mTORC1 phosphorylation sites. Interestingly, SREBPs can activate transcription of the gene encoding lipin 1 (Ishimoto et al., 2009), suggesting negative feedback regulation. Future studies will need to be done to elucidate the mechanism whereby lipin 1 regulates the abundance and the localization of mature SREBPs in the nucleus.

1.2.5.3 Regulation of SREBPs by ER Stress

The ER is a membranous organelle that plays a crucial role in protein synthesis and folding, transport, trafficking and lipid synthesis (Hotamisligil and Erbay, 2008; Ron and Walter, 2007). ER stress is a result of accumulation of unfolded or misfolded proteins in the ER lumen, which can be caused by elevated protein synthesis, calcium imbalance, nutrient overload or other stimuli that disturb ER homeostasis. ER stress triggers an adaptive response called the unfolded protein response (UPR). The UPR is mediated by three ER membrane proteins, pancreatic ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6). Under normal conditions, PERK, IRE1

and ATF6 are bound by an ER luminal chaperon glucose-regulated protein 78 (Grp78) and remain inactive. Upon ER stress, accumulation of misfolded proteins overwhelms Grp78's ability to neutralize and shield the exposed hydrophobic stretches of misfolded proteins. As a result, Grp78 dissociates from PERK, IRE1, and ATF6, leading to their activation. Once activated, these three proteins cooperatively inhibit general protein translation while promoting the expression of ER chaperone proteins and protein degradation machinery to help restore ER homeostasis. In addition, membrane expansion also occurs during ER stress to increase its protein folding capacity.

Studies have shown that ER stress activates SREBP maturation potentially to increase the synthesis of lipids needed for ER expansion (Figure 1-3) (Lee and Ye, 2004; Werstuck et al., 2001). Two mechanisms have been proposed for this process. ER stress causes depletion of Insig1 protein due to inhibition of protein synthesis, as well as the high turnover rate of Insig1 itself (Bobrovnikova-Marjon et al., 2008; Lee and Ye, 2004). This in turn promotes SREBP maturation, which can be reversed by overexpression of Insig1 (Lee and Ye, 2004). Additionally, Grp78 may also play a role in regulating ER stress-induced SREBP maturation. Overexpression of Grp78 in rat primary hepatocytes suppressed SREBP-1 maturation under ER stress conditions (Kammoun et al., 2009). Moreover, Grp78 co-immunoprecipetated with SREBP-1 in the liver of lean mice but not obese mice. These results suggest a model where, similar to PERK, IRE1, and ATF6, Grp78 binds to SREBPs and prevents them from activation. Under ER stress conditions, Grp78 dissociates from SREBPs, which

allows the ER to Golgi transport and proteolytic activation of SREBPs.

1.2.5.4 Regulation of SREBPs by Fatty Acids

Similar to the feedback inhibition of SREBP-2 by sterol, SREBP-1 is subjected to feedback inhibition by fatty acids. This effect is mainly mediated by unsaturated fatty acids (UFA) at both transcriptional and posttranslational levels (Figure 1-3). UFA inhibits *SREBP-1c* transcription by antagonizing the LXR nuclear receptor (Ou et al., 2001). In mouse, only *SREBP-1c* transcription is regulated by UFA since the *SREBP-1a* promoter does not contain an LXRE (Repa et al., 2000; Zhang et al., 2005). In human, however, an LXRE is present in the promoters of both *SREBP-1a* and *SREBP-1c* (Ye and DeBose-Boyd, 2011). Therefore, UFA can inhibit the transcription of both *SREBP-1* isoforms in human (Hannah et al., 2001).

UFA also inhibits the maturation of SREBP-1 without affecting SREBP-2 (Hannah et al., 2001). The exact mechanism underlying this effect is currently unknown, but it is proposed that Insig1 may potentially mediate this process. UFA stabilizes Insig1 protein by inhibiting its interaction with UBX domain-containing protein 8 (Ubxd8) (Lee et al., 2008). Ubxd8 recruits p97, an AAA-ATPase that extracts polyubiquitinated proteins from membrane for proteasomal degradation. The binding of Ubxd8/p97 to Insig1 is independent of sterol-induced Insig1 ubiquitination, as this interaction neither requires nor blocks Insig1 ubiquitination (Ikeda et al., 2009; Lee et al., 2008). Both ubiquitination and recruitment of p97 are required for the proteasomal degradation of Insig1 (Ikeda et al., 2009). Unlike

Insig1, UFA has no effect on Insig2 protein stability (Lee et al., 2008). Since Insig1 also regulates SREBP-2 maturation, how UFA specifically inhibits SREBP-1 maturation through Insig1 will need to be addressed in future studies.

1.2.5.5 Regulation of SREBPs by Glucagon

When blood glucose level falls, α-cells in pancreas secrete glucagon to raise the glucose level in the bloodstream (Quesada et al., 2008). Glucagon binds to the glucagon receptor, a G protein-coupled receptor, which in turn activates adenylate cyclase. Adenylate cyclase produces cyclic AMP (cAMP), which can bind to the regulatory subunit of protein kinase A (PKA) to relieve its inhibition of the catalytic subunit. Once activated in the liver, PKA inhibits glycolysis and glycogen synthesis, while stimulating glycogenolysis and gluconeogenesis. PKA also suppresses lipid synthesis partially through SREBP-1 inhibition (Figure 1-3). PKA directly phosphorylates LXR, which disrupts its dimerization with RXR and DNA binding, thus leading to decreased *SREBP-1c* transcription (Yamamoto et al., 2007). PKA can also phosphorylate mature SREBP-1 at serine residue 338 (1a) or 314 (1c), which attenuates SREBP-1 transactivation and subsequently inhibits lipogenesis (Lu and Shyy, 2006).

1.2.5.6 Regulation of SREBPs by AMPK

AMP-activated protein kinase (AMPK) is a master regulator of cellular energy homeostasis (Kahn et al., 2005). It senses intracellular energy level by detecting changes in the AMP:ATP ratio. AMPK is activated in response to

increased AMP level in a low energy state. Upon activation, AMPK inhibits energy-consuming anabolic pathways and activates energy-producing catabolic pathways to increase the cellular ATP level. In liver, AMPK suppresses lipogenesis by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), two rate-limiting enzymes in fatty acid and cholesterol biosynthesis pathways, respectively. Moreover, a recent study indicates that AMPK also inhibits SREBP maturation (Figure 1-3). AMPK directly phosphorylates SREBP-1c at serine residue 372, which in turn leads to a decreased level of the mature form and accumulation of the precursor form of SREBP-1c (Li et al., 2011). Serine to alanine mutation of the phosphorylation site abolishes the inhibitory effect of AMPK on SREBP-1c maturation. SREBP-2 is also a direct substrate of AMPK, but the phosphorylation site has not been identified. How phosphorylation on SREBPs by AMPK blocks the maturation process awaits further study.

In addition to direct inhibition, AMPK can also indirectly affect SREBP activity through crosstalk with other nutrient sensors. For example, AMPK inhibits mTORC1 activity (Kahn et al., 2005), which in turn leads to the suppression of SREBPs. Additionally, AMPK may promote the degradation of mature SREBPs by enhancing the activity of Sirtuin 1 (SIRT1), an NAD^+-dependent deacetylase (Ruderman et al., 2010; Walker et al., 2010). Mature SREBPs can be acetylated by their transcriptional coactivator CBP/p300 histone acetyltransferase (Giandomenico et al., 2003). This acetylation protects SREBPs from ubiquitination and proteasomal degradation. SIRT1 directly deacetylates mature

SREBPs, resulting in increased SREBP protein turnover and decreased lipid synthesis (Figure 1-3) (Walker et al., 2010).

1.2.5.7 Additional Signals Regulating SREBPs

Promoter analysis has provided insights into the transcriptional regulation of *SREBP-1a* and *SREBP-2*. *SREBP-1a* has a simple promoter containing an NF-κB-binding site flanked by two Sp1-binding sites (Zhang et al., 2005). NF-κB is a key transcription factor complex that mediates the expression of genes involved in immune response (Hayden and Ghosh, 2012). Consistent with this, it has been shown that in macrophages, lipopolysaccharide (LPS) challenge promotes the recruitment of NF-κB, as well as Sp1, to the *SREBP-1a* promoter. This leads to the transcriptional activation of *SREBP-1a*, which in turn promotes the expression of pro-inflammatory genes and lipogenesis (Im et al., 2011). The *SREBP-2* promoter contains an SRE, and hence is subjected to feedforward autoregulation (Sato et al., 1996). In addition, the *SREBP-2* promoter also contains a binding site for thyroid hormone receptor (TR), and is activated by TR in a ligand-dependent manner (Shin and Osborne, 2003). This is consistent with the decreased expression of SREBP-2 target gene LDL receptor (LDLR) in mice depleted with thyroid hormone (Ness et al., 1990).

Intriguingly, a recent study suggests that SREBP maturation can occur in the absence of ER to Golgi transport. In this study, the authors found that SREBP-1 plays an essential role in promoting phosphatidylcholine (PC) biosynthesis (Walker et al., 2011). Decreased PC production stimulated SREBP-

1 maturation. Interestingly, instead of affecting the ER exit of SREBP-1, low PC levels actually caused S1P and S2P to translocate from the Golgi to the ER membrane and cleave off the N-terminal mature form of SREBP-1. SREBP-2 maturation was not affected by the PC level. The mechanism underlying the differential regulation of SREBP-1 and SREBP-2 will need to be addressed in future studies.

The protein stability of mature SREBP-1c is also negatively regulated by cyclin-dependent kinase 8 (CDK8), a component of the Mediator complex. CDK8 phosphorylates SREBP-1c at threonine residue 402, a site shared by GSK3, and promotes its degradation (Figure 1-3) (Zhao et al., 2012). Interestingly, CDK8 expression is downregulated in response to insulin treatment in rat primary hepatocytes or feeding in mouse liver, consistent with its role as a suppressor of SREBP-1c.

1.2.6 SREBPs and Metabolic Diseases

The abundant supply of food high in calories and the relatively sedentary lifestyle in modern society has greatly accelerated the development and prevalence of metabolic diseases, including diabetes, obesity and nonalcoholic fatty liver disease (NAFLD). Metabolic diseases have become a major health concern in the United States. According to the CDC, over 29 million people nationwide have diabetes, and the estimated rates for adult and pediatric obesity have increased to 20-30% of the US population (Browning et al., 2004). NAFLD is another major form of metabolic disease, affecting over 30% of the general

population in the US (World Gastroenterology Organisation, 2012). NAFLD is strongly associated with diabetes and obesity. Over 90% of the obese patients with T2D are also diagnosed with NAFLD (Tolman et al., 2007). As discussed below, NAFLD greatly contributes to the disease phenotype of T2D. In addition, individuals with NAFLD have higher risk of progression towards more serious liver diseases, such as nonalcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma and liver failure (Cohen et al., 2011).

The hallmark of NAFLD is the excessive accumulation of triglyceride in hepatocytes (hepatic steatosis) (Cohen et al., 2011). As the master regulator of fatty acid and triglyceride biosynthesis, SREBP-1 plays a crucial role in the development of hepatic steatosis. Overexpression of mature SREBP-1a and SREBP-1c in mouse liver increased the hepatic TAG level by 20 fold and five fold, respectively (Shimano et al., 1996; Shimano et al., 1997a). On the other hand, deletion of *SREBP-1* attenuated fatty liver in leptin-deficient *Ob/Ob* mice, a genetic model of obesity (Yahagi et al., 2002). Moreover, the hepatic protein abundance of mature SREBP-1 was significantly elevated in *Ob/Ob* mice as well as in HFD-fed wild-type mice (Leavens et al., 2009; Shimomura et al., 1999a). This was associated with increased expression of SREBP-1 target genes and fatty acid synthesis. These results indicate that hyperactivation of SREBP-1 plays a causal role in the onset of hepatic steatosis.

The main characteristic of these metabolic diseases is insulin resistance. It is a condition where the body does not respond to insulin action efficiently due to impaired insulin signaling (Samuel and Shulman, 2012). For example, in liver,

insulin fails to activate glycogen synthesis and suppress gluconeogenesis under insulin resistant conditions, resulting in hyperglycemia. Hyperglycemia leads to hyperinsuliemia, as insulin secretion from pancreatic β-cells accelerates to compensate for the elevated blood glucose level. Interestingly, however, SREBP-1c-mediated lipogenesis pathway remains fully active, thus resulting in the triad of hyperinsulinemia, hyperglycemia and hypertriglyceridemia typically observed in insulin resistant patients (Brown and Goldstein, 2008). This “selective insulin resistance” state is different from the “complete insulin resistance” phenotype observed in liver-specific insulin receptor knockout (LIRKO) mice, where insulin can no longer activate SREBP-1c-mediated lipid synthesis in liver (Biddinger et al., 2008). As a result, LIRKO mice exhibited hyperinsulinemia and hyperglycemia, but showed low levels of plasma and hepatic TAG. Therefore, selective insulin resistance in liver contributes to more severe metabolic defects. Hypertriglyceridemia causes ectopic deposition of lipid in organs other than white adipose tissue, which further promotes insulin resistance and insulin secretion, thus leading to a vicious cycle (Chavez and Summers, 2010).

The mechanisms underlying the selective insulin resistance in liver remain unclear. One possibility is that, in addition to insulin signaling, SREBP-1c can also respond to other signals to induce lipogenesis (Ferre and Foufelle, 2010). Notably, mTORC1, which is greatly activated by the excessive nutrients (such as glucose and amino acids) available under obese and diabetic conditions (Zoncu et al., 2011), is one such candidate. ER stress could also participate in the activation of SREBP-1c under insulin resistant conditions. It has been shown that

ER stress is induced in the liver of obese, insulin-resistant rodents (Ozcan et al., 2004). Nonetheless, understanding the precise mechanisms underlying SREBP-1c regulation in liver will provide new insight into disease biology as well as therapeutic targets for metabolic disorders.

1.3 Thesis Overview

PASK is proposed to play an important role in regulating cellular metabolism in response to nutrient status and other environmental conditions. *Pask*$^{-/-}$ mice are resistant to HFD-induced metabolic disorders. Interestingly, *Pask*$^{-/-}$ mice showed almost complete protection from hepatic steatosis, suggesting a potential role of PASK in regulating hepatic lipid metabolism. Hepatic steatosis is a major component of the metabolic syndrome and makes detrimental contributions to disease progression. However, the molecular mechanisms underlying hepatic steastosis remain unclear. Therefore, in this dissertation, we aimed to determine the mechanism whereby PASK regulates lipid metabolism in liver, as well as to explore the therapeutic potential of PASK in metabolic disorders. In Chapter 2, we describe the identification of SREBP-1 as a functional downstream target of PASK in mediating hepatic lipogenesis. We also show that pharmacological inhibition of PASK improved hepatic and whole-body dyslipidemia in animal models of diet-induced metabolic diseases. Additionally, we present two preliminary studies where we (1) examine the mechanism whereby PASK activates SREBP-1; and (2) investigate the upstream signaling pathway leading to SREBP-1 activation by PASK. Results from these studies not

only further our understanding of the physiological functions of PASK, but also provide valuable insight into the pathogenesis and treatment of NAFLD and other metabolic disorders.

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CHAPTER 2

PAS KINASE DRIVES LIPOGENESIS THROUGH SREBP-1 MATURATION

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PAS Kinase Drives Lipogenesis through SREBP-1 Maturation

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SUMMARY

Elevated hepatic synthesis of fatty acids and triglycerides, driven by hyperactivation of the SREBP-1c transcription factor, has been implicated as a causal feature of metabolic syndrome. SREBP-1c activation requires the proteolytic maturation of the endoplasmic-reticulum-bound precursor to the active, nuclear transcription factor, which is stimulated by feeding and insulin signaling. Here, we show that feeding and insulin stimulate the hepatic expression of *PASK*. We also demonstrate, using genetic and pharmacological approaches, that PASK is required for the proteolytic maturation of SREBP-1c in cultured cells and in the mouse and rat liver. Inhibition of PASK improves lipid and glucose metabolism in dietary animal models of obesity and dyslipidemia. Administration of a PASK inhibitor decreases hepatic expression of lipogenic SREBP-1c target genes, decreases serum triglycerides, and partially reverses insulin resistance. While the signaling network that controls SREBP-1c activation is complex, we propose that PASK is an important component with therapeutic potential.

INTRODUCTION

Excessive synthesis and storage of lipids is a prominent feature of the current epidemic of metabolic disorders, including obesity, diabetes, and their comorbidities. Upon feeding, fatty acids and triglycerides are synthesized primarily in the liver and adipose tissue in response to insulin signaling, and then are either stored locally or exported to other tissues for use in ATP production. The Sterol Regulatory Element Binding Protein (SREBP-1c) transcription factor is a principal regulator of lipogenesis in these two tissues (Horton et al., 2002; Rosen et al., 2000). Upon activation, SREBP-1c stimulates expression of the entire enzymatic pathway that converts acetate to fatty acids and their esterification to triacylglycerol (TAG) (Horton et al., 2003). Hyperactivation of SREBP-1c has been implicated in promoting pathological fat synthesis and driving features of metabolic syndrome, including hepatic lipid accumulation (or steatosis), dyslipidemia, and insulin resistance (Brown and Goldstein, 2008).

Although it is often pathological in modern humans, SREBP-1c activation in response to feeding is a normal physiological response that enables the storage of excess energy in the stable and compact TAG form. SREBP-1c activation by feeding occurs predominantly in response to insulin, which acts at multiple regulatory steps. Transcription of the *SREBP-1c* mRNA is strongly induced by insulin via a mechanism involving the LXR transcription factor (Chen et al., 2004; DeBose-Boyd et al., 2001; Repa et al., 2000; Schultz et al., 2000; Yoshikawa et al., 2001) and *SREBP-1c* autoregulation (Amemiya-Kudo et al., 2000; Chen et al., 2004). Insulin also acts, through GSK-3β inhibition and potentially through Lipin1 phosphorylation, to extend the otherwise very short half-life of active SREBP-1c (Harris et al., 2007; Péterfy et al., 2010; Peterson et al., 2011; Sundqvist et al., 2005). One of the most important mechanisms underlying SREBP-1c activation by insulin signaling, however, is the proteolytic maturation of the endoplasmic reticulum (ER) membrane-embedded SREBP-1c precursor into the active and nuclear mature SREBP-1c transcription factor (Hegarty et al., 2005).

Analogous to what has been described for its better-characterized paralog, SREBP-2, maturation of SREBP-1c is thought to occur through the regulated translocation of the precursor to the Golgi, where it is cleaved sequentially by two proteases, liberating the mature form from its two transmembrane segments (Horton et al., 2002; Raghow et al., 2008). The regulatory pathway linking insulin and SREBP-1c maturation is incompletely understood, but has been shown to require the canonical PI3K/Akt pathway (Krycer et al., 2010; Yellaturu et al., 2009a). More recently, evidence has shown that insulin-responsive SREBP-1c activation also requires the mechanistic Target of Rapamycin Complex 1 (mTORC1) (Düvel et al., 2010; Li et al., 2010; Porstmann et al., 2008). Although part of the effect of insulin/Akt on SREBP-1c maturation appears to depend on the regulation of *INSIG2* gene expression (Yecies et al., 2011; Yellaturu et al., 2009b), the mechanism(s) underlying Akt/mTORC1's effect on SREBP-1c proteolytic maturation have not been identified.

PAS kinase (PASK) is an evolutionarily conserved serine/threonine kinase, and we previously proposed that it plays an important role as a nutrient-responsive metabolic regulator (Hao and Rutter, 2008). Mice lacking the *PASK* gene (*Pask*$^{-/-}$) exhibit a number of tissue-specific metabolic abnormalities, but the most profound phenotype observed to date is decreased susceptibility to hepatic lipid infiltration in animals challenged with a high-fat diet (HFD) (Hao et al., 2007). Hepatic steatosis is now recognized to be a common and devastating component of metabolic syndrome (Cohen et al., 2011). However, the molecular mechanisms underlying hepatic steatosis are unknown and no therapies are currently approved for its treatment. Given the profound *Pask*$^{-/-}$ phenotype, the importance of this process in human disease, and the potential to discover new therapeutic targets, we sought to identify the mechanism whereby PASK regulates hepatic lipid metabolism.

RESULTS

PASK Stimulates Hepatic Lipogenesis by Activating SREBP-1c

Since *Pask*$^{-/-}$ mice are protected from HFD-induced hepatic steatosis, we initially compared the hepatic transcriptional profiles of lipogenic genes from *Pask*$^{-/-}$ and wild-type (WT) mice in both fasted and fed states. The expression levels of the genes encoding glycerol-3-phosphate acyltransferase (*Gpat1*), fatty acid synthase (*Fasn*), and acetyl-coA carboxylase (*Acc1*) were all similarly low in the fasted state in WT and *Pask*$^{-/-}$ livers (Figures 1A–1C). Although expression of these genes increased substantially in WT mice upon feeding, this induction was blunted in *Pask*$^{-/-}$ mice (Figures 1A–1C). This feeding-dependent effect led us to test the expression of these same genes in fasted and fed mice that were maintained on a normal chow diet (NCD). As with the HFD, the expression levels of the lipogenic genes were similar in WT and *Pask*$^{-/-}$ mice in the fasted state. Refeeding for 6 or 8 hr, however, significantly increased the expression of *Gpat1*, *Fasn*, and *Acc1* in WT liver, and this increase was absent or blunted in *Pask*$^{-/-}$ mice (Figures 1D–1F).

We previously observed that PASK activity was nutrient responsive in cultured cells. This led us to hypothesize that PASK expression or activity might also be stimulated in the liver upon feeding, which might then be required for NCD-induced lipogenic gene expression. We therefore measured the hepatic *Pask* mRNA levels in fasted and fed mice. HFD-fed WT mice showed an increase in *Pask* expression upon feeding (Figure 1G). An even larger increase was observed in NCD-fed animals upon 2 hr of feeding following a fast (Figure 1H). Interestingly, by 6 hr of feeding, the *Pask* mRNA abundance had returned to the fasted level. This acute hepatic induction of *Pask* by feeding is likely to be a cell-autonomous response to insulin, as we observed induction of *Pask* mRNA by insulin in WT primary hepatocytes (Figure 1I).

To further investigate the mechanisms whereby PASK deficiency led to reduced expression of lipogenic genes, we employed HepG2 human hepatoma cells treated with insulin, which is a major hormonal mediator of the response of lipogenic gene expression to feeding. *PASK* knockdown by three different small interfering RNAs (siRNAs; denoted as siA, siB, and siC) in HepG2 cells (Figure 2A) caused decreased expression of *GPAT1* and *SCD1* upon insulin treatment (Figure 2B). Unlike the liver, however, HepG2 cells seem to have a basal level of lipogenic gene expression that is PASK dependent. In the absence of insulin, *PASK* knockdown caused decreased expression of *GPAT1* and *SCD1* in HepG2 cells (Figure S1A). The fact that acute *PASK* knockdown in HepG2 cells recapitulates the regulation of gene expression observed in vivo in *Pask*$^{-/-}$ liver suggests a direct role for PASK in regulating lipogenesis.

To determine whether this change in mRNA abundance is due to transcriptional control, we examined the effects of *PASK* knockdown on luciferase activity from a reporter gene driven by the promoter of either *GPAT1* (Yoshida et al., 2009) or *SCD1* (Bené et al., 2001). As shown in Figures S1B and S1C, *PASK* knockdown decreased the activity of both the *GPAT1* and *SCD1* promoters. Because SREBP-1c is one of the major feeding- and insulin-responsive regulators of GPAT1, SCD1, and the other components of the fatty acid and TAG biosynthetic pathway, we analyzed the effects of *PASK* knockdown on a luciferase reporter gene containing isolated SREBP-binding sites (Dooley et al., 1998). *PASK* knockdown caused a modest reduction in SREBP activity in serum-starved conditions, which became more pronounced upon insulin treatment (Figure 2C). One potential explanation for the loss of basal and insulin-stimulated SREBP-1 activation upon *PASK* knockdown is that the Akt/mTORC1 pathway, which is required for SREBP-1 activation, is impaired by *PASK* knockdown. However, the phosphorylation states of Akt and S6K (indicative of Akt and mTORC1 activity, respectively) were both unchanged by *PASK* knockdown (Figure 2D), demonstrating that the effects of PASK on SREBP-1 activation occur without effects on Akt and mTORC1 activity. Over many independent experiments, *PASK* knockdown with each of these three different siRNAs caused a significant impairment in the fold-induction of SREBP activity by insulin (siA: $n = 15$, $p = 0.003$; siB: $n = 16$, $p < 0.0001$; siC: $n = 9$, $p = 0.01$). Taken together, these data support a model wherein SREBP-1c is a major mediator of the transcriptional effects of PASK on the regulation of lipid biosynthesis, and PASK is required for the full effect of insulin on SREBP-1c induction.

PASK Promotes SREBP-1 Maturation

SREBP-1c is profoundly regulated by insulin at multiple levels, as described above. Given the requirement of PASK for the normal regulation of SREBP-1c, we first assessed whether it is also required for its proteolytic maturation. We examined this directly by monitoring the levels of the precursor and mature forms of SREBP-1c. We also examined the maturation of SREBP-1a, which is derived from the same gene as SREBP-1c but is the product of an alternative promoter (Hua et al., 1995). Treatment of cells with either one of two *PASK*-specific siRNAs (siA and siB), but not the control siRNA, blunted the insulin-responsive maturation of SREBP-1a and SREBP-1c (Figures 3A and S2A). As before, *PASK* knockdown impaired SREBP-1 processing, but had no effect on Akt or S6K phosphorylation (Figures 3A and S2A). The insulin-responsive proteolytic maturation of SREBP-1c is also observed upon feeding in rodent liver. In WT mice maintained on an NCD, we observed a significant increase in the abundance of mature SREBP-1c in the fed state relative to

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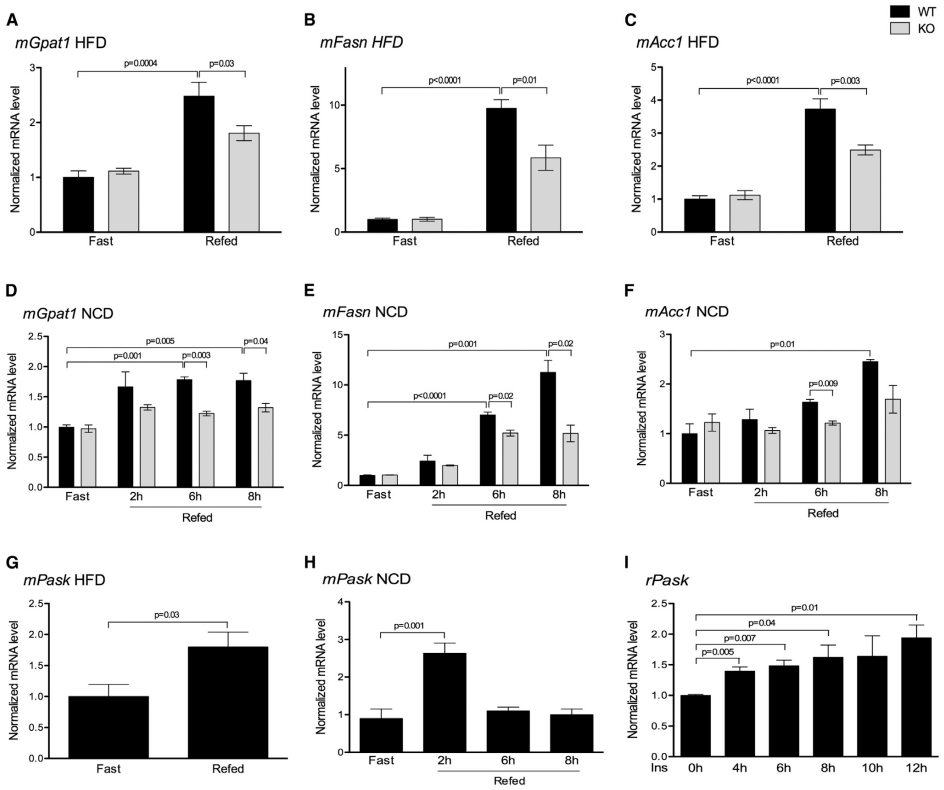

Figure 1. PASK Is Feeding Induced and Is Required for Normal Feeding-Dependent Induction of Lipogenic Gene Expression.

(A–H) WT and *Pask*$^{-/-}$ (KO) mice on the C57/BL6J background were maintained on a 60% HFD (A–C and G) for 8 weeks (n ≥ 4) or an NCD (D–F and H) for 12 weeks (n = 3). Before harvesting, mice were fasted for 24 hr or fasted for 24 hr and refed either an HFD for 12 hr (A–C and G) or an NCD for the indicated times (D–F and H). Livers were harvested and the mRNA levels of the indicated genes were measured by quantitative RT-PCR (qRT-PCR) and normalized to *Cyclophilin A* mRNA. Data shown are the average ± SEM, with the "WT fasted" value set as one.

(I) Rat primary hepatocytes were serum starved overnight and incubated with 25 nM insulin for the indicated times. *Pask* mRNA was measured by qRT-PCR and normalized to *r36B4*. Data shown are the average of n = 3 ± SEM. The value of the 0 hr group was set as one.

See also Tables S1 and S2.

the fasted state (Figure 3B). This induction was severely blunted in *Pask*$^{-/-}$ mice (Figure 3B). Feeding-induced maturation of SREBP-1c was also observed in WT mice maintained on an HFD, but was essentially absent in *Pask*$^{-/-}$ mice (Figure 3C).

The INSIG proteins are negative regulators of SREBP-1 maturation and are regulated at many levels, including transcription and mRNA stability (Gong et al., 2006; Yabe et al., 2002, 2003; Yang et al., 2002; Yellaturu et al., 2009b). Alterations in the expression of *Insig2a* have been specifically implicated in mediating the effects of insulin on SREBP-1c processing in hepatic tissue (Yabe et al., 2003; Yecies et al., 2011; Yellaturu et al., 2009b). However, it appears that the effects of *PASK* knockdown on SREBP-1c maturation are not due to altered *INSIG2* expression, as this gene is not induced in HepG2 cells upon *PASK* knockdown (Figure 3D). In fact, *INSIG2* expression actually is decreased by two out of three *PASK*-targeted siRNAs, perhaps as a compensatory mechanism that the cell employs to attempt to restore SREBP-1c maturation. We measured the *Insig1*, *Insig2a* and *Insig2b* mRNAs in WT and *Pask*$^{-/-}$ liver under fed and fasted conditions in mice maintained on either the NCD or HFD. Again, under no condition did we observe an increase in *Insig2a* expression (Figures 3E and 3F).

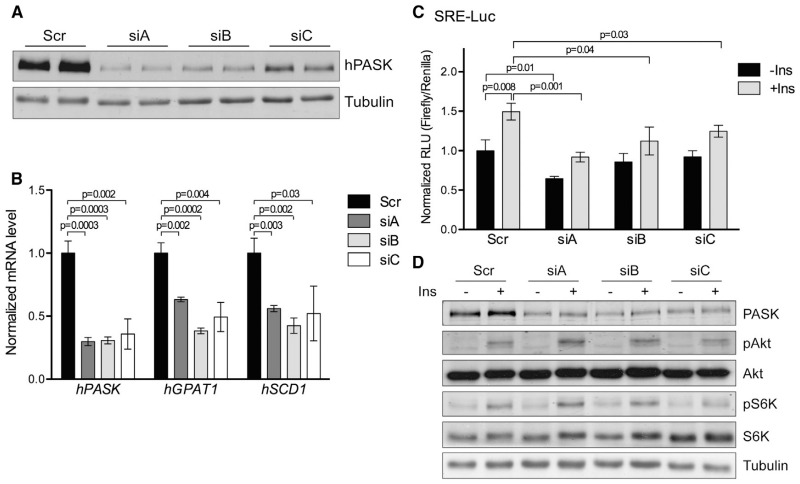

Figure 2. PASK Is Required for Full SREBP-1 Activity

(A) HepG2 cells were treated with scrambled or *PASK*-specific siRNA (siA, siB, or siC) and subjected to immunoblot for PASK.

(B) *PASK*-silenced HepG2 cells were serum starved overnight and stimulated with 100 nM insulin for 6 hr. The mRNA levels of the indicated genes were measured by qRT-PCR and normalized to *Tubulin* mRNA levels. Data shown are the average of n = 3 ± SEM, with the Scr value set as one.

(C) *PASK*-silenced HepG2 cells were transfected with SRE-Luc reporter, serum starved overnight, and stimulated with 100 nM insulin for 6 hr as indicated. Firefly and Renilla luciferase were assayed using the Dual-Reporter Luciferase Assay System. Data shown are the average of n = 3 ± SD, with the "scr −ins" value set as one.

(D) Following the luciferase assay, cell lysates from (C) were subjected to immunoblot to determine the phosphorylation state and abundance of the indicated proteins.

See also Figure S1.

We also did not observe an increase in expression of the other INSIG isoforms, *Insig1* and *Insig2b*, in *Pask*$^{-/-}$ mice (Figures S2B and S2C). We therefore conclude that PASK promotes SREBP-1 maturation, but does so independently of effects on INSIG gene expression.

We also addressed whether PASK might regulate the stability or transcriptional activation potential of mature, nuclear SREBP-1 (other known mechanisms of SREBP-1 control). We expressed a truncated form of SREBP-1a, or GFP as a control, and asked whether *PASK* knockdown had any effect on SRE-driven luciferase activity. As shown in Figure 3G, *PASK* knockdown with siB in GFP-control cells led to decreased luciferase activity in both the presence and absence of insulin. Expression of the mature SREBP-1a, however, caused luciferase activity to become completely insensitive to *PASK* knockdown (Figure 3G). We were concerned that expression of this ectopic transcription factor might have overwhelmed the PASK regulatory system and the PASK insensitivity was due to an overexpression artifact. Therefore, we expressed the full-length, precursor SREBP-1a and found that *PASK* knockdown retained the ability to suppress luciferase activity (Figure 3G). To achieve a similar transcriptional response, we expressed much more full-length SREBP-1a, which requires processing for activation. In spite of this, the full-length SREBP-1a was regulated by PASK, whereas the mature form of SREBP-1a was not. We thus conclude that the activity of the nuclear, mature form is resistant to *PASK* knockdown. *PASK* knockdown with siA, which is more effective at silencing *PASK* and decreasing SREBP-1 activity (Figure 2), followed the same pattern, although *PASK* knockdown still caused a small but significant decrease in SREBP activity even when the mature SREBP-1a was expressed (Figure S2D). This was likely due to endogenous SREBP-1, whose activity remained PASK dependent. As before, the activity of over-expressed full-length, precursor SREBP-1a was PASK dependent. The loss of SREBP-1 activity upon *PASK* knockdown again occurred in the absence of any effect on insulin or mTORC1 signaling (Figures 3G and S2D). We initially attempted the above experiment with SREBP-1c, which has much weaker transcriptional activation potential than SREBP-1a (Shimano et al., 1997a), but we were unable to express enough full-length precursor SREBP-1c to achieve comparable luciferase activity. This technical issue prevented a rigorous interpretation of the analogous experiment with SREBP-1c.

Finally, we sought to address whether the transcriptional control of SREBP-1c might depend upon PASK. This is a difficult question to answer, however, because SREBP-1c stimulates the expression of its own promoter (Amemiya-Kudo et al., 2000; Chen et al., 2004). Therefore, posttranscriptional effects on

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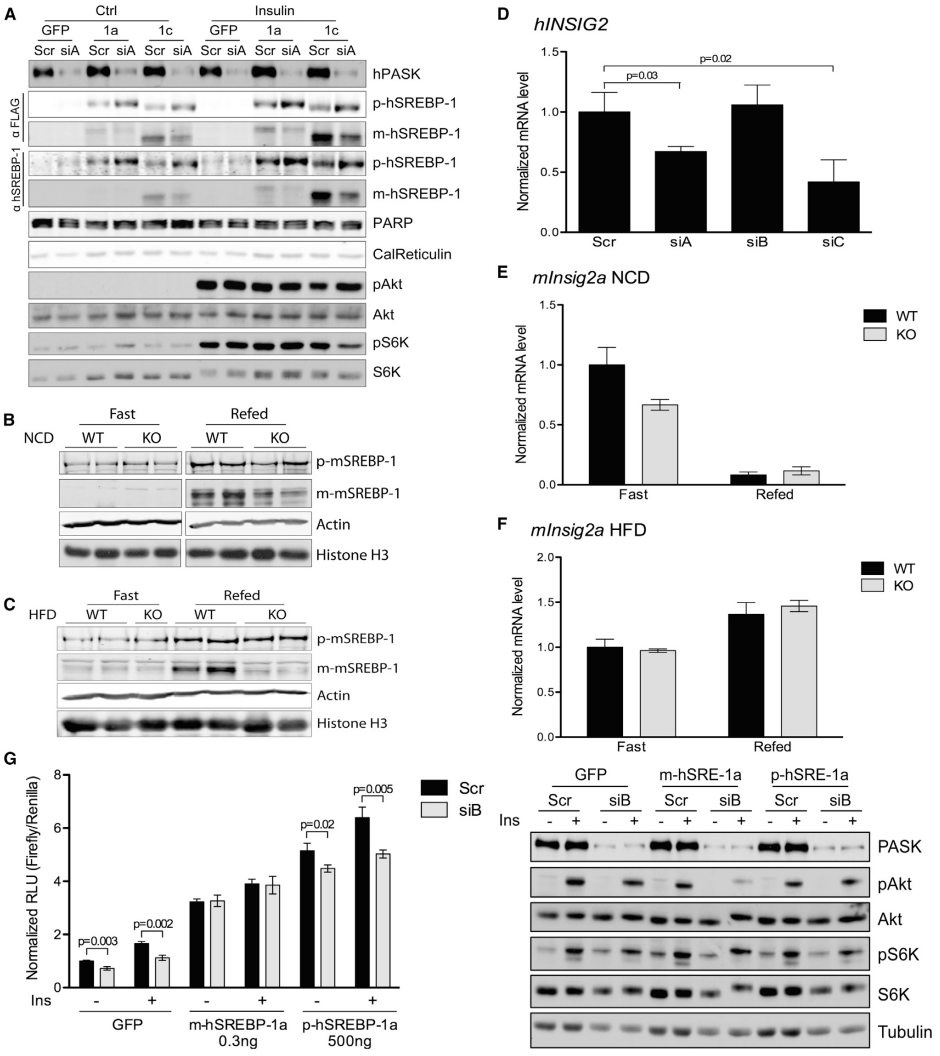

Figure 3. PASK Promotes SREBP-1 Maturation

(A) *PASK*-silenced HepG2 cells expressing GFP, 3xFlag-tagged precursor SREBP-1a, or SREBP-1c were serum starved overnight and stimulated with 100 nM insulin for 6 hr as indicated. Whole-cell lysates and nuclear extracts were subjected to immunoblot to determine the phosphorylation state and abundance of the indicated proteins.

(B and C) Livers were harvested from WT or *Pask*$^{-/-}$ (KO) mice maintained on an NCD (B) or HFD (C) as described in Figure 1, except that mice were refed for 8 hr (B) or 6 hr (C). Whole-liver lysates and nuclear extracts were subjected to immunoblot to determine the abundance of the indicated proteins.

(legend continued on next page)

SREBP-1c activity will indirectly result in alterations to the *SREBP-1c* mRNA level. As expected, we observed that the *SREBP-1c* mRNA was decreased in *Pask*$^{-/-}$ liver (Table S1) in parallel with the decrease in SREBP-1c target genes described above (Figure 1). Inhibition of PASK caused a modest reduction in the activity of the *mSREBP-1c* promoter as determined using a luciferase reporter assay in rat primary hepatocytes stimulated with insulin (Figure S2E). This effect of PASK on promoter activity was dependent on SREBP-1 autoregulation, however, as a promoter in which the SREBP-binding site had been mutated showed no response to PASK inhibition (Figure S2E). We also observed a modest decrease in the level of the *SREBP-1a* mRNA (Table S1), but this is unlikely to be a major contributor to the expression of lipogenic genes in the liver because it is expressed at a much lower level than *SREBP-1c* (Shimomura et al., 1997). We observed no consistent alteration in the expression of *SREBP-2* or its target genes in *Pask*$^{-/-}$ mice maintained on either the NCD or HFD (Tables S1 and S2). Taken together, these experiments suggest that in the absence of transcriptional control, PASK regulates the posttranslational maturation of SREBP-1 in response to insulin. However, we cannot exclude the possibility that PASK might also regulate SREBP-1c transcriptionally.

Pharmacological Inhibition of PASK Leads to Decreased SREBP-1 Activity

To facilitate the assessment of the importance of PASK for regulating SREBP-1c activity and lipid homeostasis in vivo, we developed a series of PASK inhibitors. Among them, we selected for further study two highly selective and potent inhibitors: BioE-1115 and BioE-1197 (Figure 4A). To assess their in vitro specificity, we measured the activity of PASK and 50 other protein kinases, selected to represent the breadth of the human kinase family, in the presence of BioE-1115 or BioE-1197. As shown in Figure 4B, BioE-1115 specifically inhibits PASK, with an IC_{50} of ~4 nM. Second to PASK, casein kinase 2α was the kinase most potently inhibited by BioE-1115, having an IC_{50} of ~10 μM. This is interesting because of the unexpected structural similarities between the kinase domains of PASK and casein kinase 2 (Kikani et al., 2010). In spite of this similarity, however, BioE-1115 was roughly 2,500-fold more potent as an inhibitor of PASK than of casein kinase 2α2 (Figure 4B). BioE-1197 showed similar specificity for PASK versus the same 50 protein kinases. As shown in Figure S3A, even when we used an extremely high concentration of BioE-1197 (100 μM), the majority of the 50 kinases were either unaffected or only modestly inhibited. PASK, however, was essentially completely inactivated and eight other kinases were also substantially inhibited. In full dose-response inhibition curves for these nine BioE-1197-sensitive kinases, all but two were inhibited poorly, with an IC_{50} of >50 μM (Figure S3B). Similar to what we observed with BioE-1115, casein kinase 2α was inhibited by BioE-1197, but with a ~1,000-fold higher IC_{50} compared with PASK (Figure S3B). For subsequent use as a control, we also synthesized an enantiomer of BioE-1197, which was ~100-fold less potent at inhibiting PASK in vitro (IC_{50} = 870 nM; Figure 4C).

We next examined the efficacy of BioE-1115, BioE-1197, and BioE-1428 for PASK inhibition in cultured cells. We tested this by virtue of the ability of PASK to autophosphorylate at Thr-307, which we have demonstrated to be solely dependent upon PASK activity (Figure S3C). In the presence of either BioE-1115 or BioE-1197, we observed a dose-dependent loss of PASK phosphorylation, with an IC_{50} of ~1 μM (Figure 4D). As expected, the enantiomer BioE-1428 was less effective at PASK inhibition in cells, with an IC_{50} of >10 μM (Figure 4D).

Having validated that BioE-1115 and BioE-1197 are potent and specific inhibitors of PASK, we were then able to use them to probe the acute effects of PASK inhibition. Specifically, we sought to determine whether pharmacological PASK inhibition, like *PASK* knockdown and *PASK* deletion, leads to impaired SREBP-1 activity. We measured SRE-driven luciferase activity in the presence of varying concentrations of BioE-1115 and BioE-1197. We observed a significant reduction in SREBP activity at all concentrations above 10 μM for BioE-1115 and 3 μM for BioE-1197 (Figure 5A), without any observable effects on cell morphology or growth rate. This effect is likely to be specific for PASK inhibition, because treatment of cells with BioE-1428 caused only a modest reduction of SREBP activity even at the highest concentration tested, 50 μM (Figure S3D). As with *PASK* knockdown, neither compound impaired the phosphorylation of Akt or S6K in response to insulin (Figures 5B and S3E), demonstrating again that the loss of SREBP-1 activity is not due to a loss of canonical insulin signaling.

If BioE-1115 and BioE-1197 suppress SREBP-1 activity through inhibition of PASK, they should act by blocking SREBP-1 proteolytic activation, as was observed with *PASK* knockdown in cells and in the liver of *Pask*$^{-/-}$ mice. Both BioE-1115 and BioE-1197 suppressed SREBP-1c maturation, as evidenced by a decrease in the mature/precursor SREBP-1c ratio, at 30 and 50 μM concentrations (Figures 5C and S3F). In contrast, BioE-1428 had no effect at 30 μM and the effect at 50 μM was weaker than observed with BioE-1197 (Figure S3F). When quantified over five independent experiments, BioE-1197 was significantly more efficacious at suppressing SREBP-1c maturation than BioE-1428 (Figure S3G). In summary, both *PASK* knockdown and pharmacological inhibition led to impaired SREBP-1 activity, and both manipulations impacted the maturation step. These complementary data support the hypothesis that PASK is required for the normal maturation and activation of SREBP-1.

(D) HepG2 cells were treated and human *INSIG2* mRNA levels were analyzed as in Figure 2B. Data shown are the average of n = 3 ± SEM, with the Scr value set as one.
(E and F) Mouse *Insig2a* mRNA levels in livers of mice (from B and C) fed an NCD (E) or HFD (F) were measured as described in Figure 1. Data shown are the average of n = 3 ± SEM, with the "WT fasted" value set as one.
(G) Left: SRE-Luc activity was measured in *PASK*-silenced HepG2 cells expressing GFP or mature or precursor SREBP-1a along with SRE-Luc. Right: following the luciferase assay, cell lysates were subjected to immunoblot to determine the phosphorylation state and abundance of the indicated proteins.
See also Figure S2.

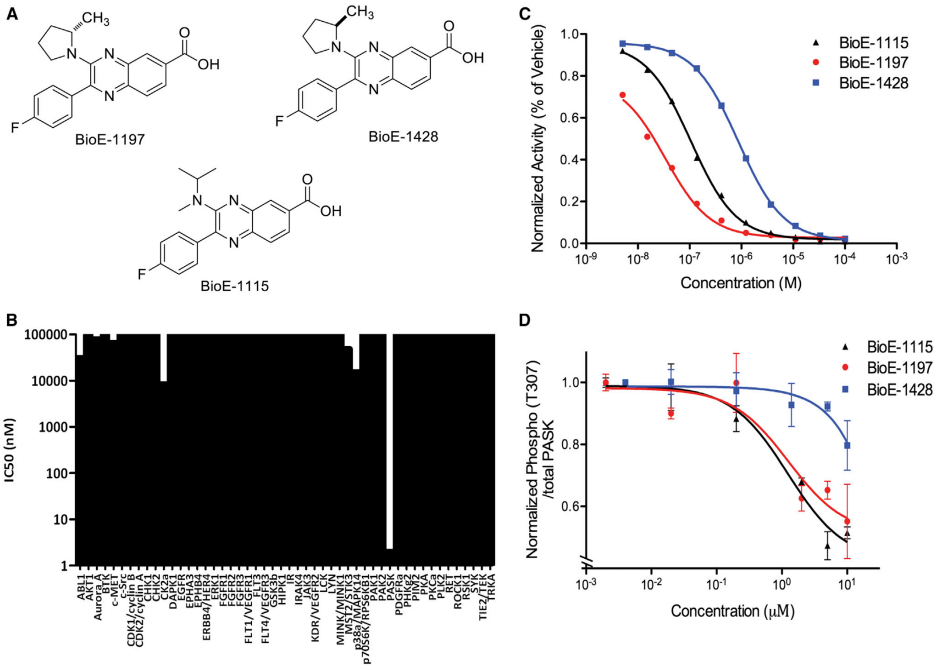

Figure 4. BioE-1115 and BioE-1197 are PASK-Specific Inhibitors

(A) Chemical structures of BioE-1115, BioE-1197, and BioE-1428.

(B) The activity of the indicated kinases was measured in the presence of either vehicle or BioE-1115, and IC_{50} values were measured.

(C) Purified PASK protein kinase activity was assayed as in (B) in the presence of the indicated concentrations of BioE-1197, BioE-1428, or BioE-1115, and the percentage of vehicle-treated activity was determined as indicated.

(D) HEK293 cells expressing a Flag-tagged PASK protein were treated with the indicated concentrations of BioE-1115, BioE-1197, or BioE-1428. PASK activity was analyzed by ELISA with both phospho-Akt substrate antibody and pan-PASK antibody. The quantitated phospho/total PASK signal is plotted ± SEM.

See also Figure S3.

We next tested whether PASK inhibition would lead to a decrease in the synthesis of fatty acids and triglycerides, as would be predicted based on the impairment in SREBP-1c activity. We measured the incorporation of ^{14}C from ^{14}C-acetate into lipid soluble material, which is primarily triglyceride under these conditions. Treatment with 10 μM BioE-1197 caused a ~60% decrease in ^{14}C incorporation into lipids (Figure 5D), suggesting that the impaired SREBP-1 activity is manifest at the level of lipogenesis.

Rat primary hepatocytes have been used extensively in studies of insulin-responsive SREBP-1c activation and lipogenesis. We previously observed that *PASK* gene expression was stimulated by insulin (Figure 1I). Therefore, we explored whether PASK inhibition would have any effect on the insulin-responsive expression of SREBP-1c target genes in these cells. Primary hepatocytes were isolated, plated, pretreated with DMSO or BioE-1197 for 16 hr, and then stimulated with either insulin or vehicle over a 12 hr time course. Insulin caused a significant increase in the mRNAs encoding fatty acid synthase and glucokinase (*Fasn* and *Gck*, respectively; Figures 5E and 5F). This increase was either blunted or abolished by the PASK inhibitor. In these cells, BioE-1197 caused a significant decrease in the mRNA encoding *SREBP-1c* under both basal and insulin-stimulated conditions (Figure 5G). Finally, we measured the incorporation of ^{14}C from ^{14}C-acetate into lipids. Insulin caused a significant increase in lipogenesis in vehicle-treated hepatocytes, which was completely abolished by treatment with BioE-1197 (Figure 5H).

PASK Inhibition Reduces SREBP-1c Activity and Hypertriglyceridemia in Rodents

We previously observed that PASK deficiency in mice leads to protection against many of the pathological effects of an HFD (Hao et al., 2007). Most prominently, in the previous study we

found that *Pask*$^{-/-}$ mice were protected from the severe diet-induced hepatic steatosis observed in WT mice. This lipid phenotype was accompanied by a decrease in the expression of the lipogenic program driven by SREBP-1c (Figure 1). Because we conducted these studies using a constitutively deleted allele of *PASK*, however, we were concerned that this phenotype might be due to adaptation or a developmental abnormality. Therefore, we initiated studies to address the effects of acute inhibition of PASK in adult animal models of metabolic disease. These studies were conducted with BioE-1115 due to the more favorable in vivo pharmacological and pharmacokinetic properties of this PASK inhibitor relative to BioE-1197 (Figure S4A).

WT Sprague-Dawley (SD) rats at 12 weeks of age were fed for 2 weeks with either an NCD or a high-fructose diet (HFrD), which is known to promote dyslipidemia and insulin resistance (Hwang et al., 1987). Those fed the HFrD were then orally dosed with vehicle or a range of doses of BioE-1115 once a day for 1 week. Following this 1-week treatment, we harvested livers from these rats after they were refed, and examined the mRNA levels of SREBP-1c target genes. As expected, the SREBP-1c target genes were upregulated in animals fed the HFrD relative to control (Figures 6A and S4B–S4D). Among the HFrD group, rats treated with either 1 or 3 mg BioE-1115 per kilogram of body weight (mg/kg) showed no difference from vehicle-treated rats. Those treated with 10, 30, and 100 mg/kg, however, showed a dose-dependent suppression of the expression of *Gpat1*, *Fasn* (Figure 6A), and all other SREBP-1c target genes analyzed (Figures S4B–S4D). SREBP-1 maturation in the liver was also suppressed in BioE-1115-treated rats at these three doses (Figure 6B), which is consistent with what we observed in *Pask*$^{-/-}$ mouse liver. However, the SREBP-2 target genes did not follow this pattern consistently (Figures S4E–S4I). In fact, HMG-CoA synthase 1 and *SREBP-2* itself both followed the opposite pattern (Figures S4E and S4J), being induced in a dose-dependent manner by BioE-1115. This is particularly intriguing given that mice lacking *SREBP-1* frequently die in utero, but those that survive exhibit elevated expression of *SREBP-2* and its target genes, implying some sort of compensatory mechanism between the two transcription factors (Shimano et al., 1997b). One SREBP-2 target gene, that encoding HMG-CoA reductase, was significantly decreased in BioE-1197-treated animals (Figure S4F). Interestingly, this is the one SREBP-2 target gene that was also significantly underexpressed in *Pask*$^{-/-}$ mice (Table S2). Both *SREBP-1c* and *SREBP-1a* mRNA were modestly decreased at the highest doses of BioE-1115 (Figures S4K and S4L).

In addition to the marked change in SREBP-1c target gene expression, we also observed a reversal of the dyslipidemic features associated with an HFrD, as BioE-1115 treatment caused a decrease in hepatic TAG (Figure 6C). Similarly, serum TAG was also decreased in a dose-dependent manner by BioE-1115 administration (Figure 6D), whereas serum cholesterol was basically unaffected (Figure 6E). Due to the normalization of triglyceride concentrations, we speculated that BioE-1115 might have salutary effects on glucose homeostasis as well. Indeed, the PASK inhibitor caused a significant decrease in serum glucose (Figure 6F). This drop in glucose levels was almost certainly a consequence of increased insulin sensitivity rather than increased insulin secretion, as serum insulin levels were slightly lower (rather than higher) in drug-treated animals (Figure 6G). A calculated measure of insulin resistance, termed homeostasis model assessment-estimated insulin resistance (HOMA-IR), was decreased in a dose-dependent manner by BioE-1115 administration (Figure 6H). Neither dose of BioE-1115 caused a significant change in either liver weight or body weight (Figures 6I and 6J), suggesting that the effects on lipid and glucose homeostasis were not due to overt toxicity.

We next performed a study of similar design except that the HFrD-fed rats were treated for 90 days with either vehicle or BioE-1115. The effect on SREBP-1c target gene expression was enhanced with the longer treatment time. At 3 mg/kg BioE-1115, expression of both *Fasn* and *Acc1* was significantly suppressed by PASK inhibition, and at 10, 30, and 100 mg/kg, expression of these genes was restored to that in NCD-fed animals (Figure S5A). The effect on SREBP-1c activity appears to be specific for the liver, as there were no significant differences in the expression of *SREBP-1c* or its target genes in abdominal fat (Figure S6A) or in gastrocnemius muscle (Figure S6B) upon BioE-1115 treatment. After 90 days of treatment and at the interim time point of 45 days, we observed a significant dose-responsive decrease in serum triglycerides (Figures S5B and S5C). As in the shorter dosing period, BioE-1115 decreased serum glucose levels as measured by glycated hemoglobin (HbA1c), which is a measure of chronic glycemia (Figures S5D and S5E). After both 51 and 90 days of dosing, there was no drug-dependent difference in body weight between any of the vehicle or treatment groups (Figures S5F and S5G).

DISCUSSION

The regulation of SREBP-1c is complex in terms of both the regulatory stimuli involved and how these stimuli impinge upon SREBP-1c activity (Raghow et al., 2008). The most widely studied and understood stimulus that promotes SREBP-1c activation is insulin, and various components of its downstream signaling pathway, including Akt and mTOR, have been implicated (Jeon and Osborne, 2012). These related mechanisms combine to elicit a profound activation of SREBP-1c in response to feeding. We show herein that PASK is transcriptionally induced by feeding in vivo in the liver and is also induced in a cell-autonomous manner by insulin in primary hepatocytes. This induction appears to be related to SREBP-1c activation, as PASK is required for normal feeding and insulin-responsive SREBP-1c activity. Inhibition of PASK also impairs insulin-responsive lipid biosynthesis, which is driven by SREBP-1c. We demonstrated this requirement for PASK in SREBP-1c activation in four model systems and by using three distinct modalities to block PASK activity. SREBP-1c-driven transcription is impaired by genetic deletion of *PASK* in mice fed either an NCD or HFD, pharmacological inhibition and siRNA-mediated knockdown in cultured HepG2 cells, and pharmacological inhibition in both rat primary hepatocytes and HFrD-fed rats. Not only do these disparate manipulations cause the same physiological effect, they also appear to act via the same biochemical mechanism. This provides a compelling argument that PASK is an important regulator of SREBP-1c activation.

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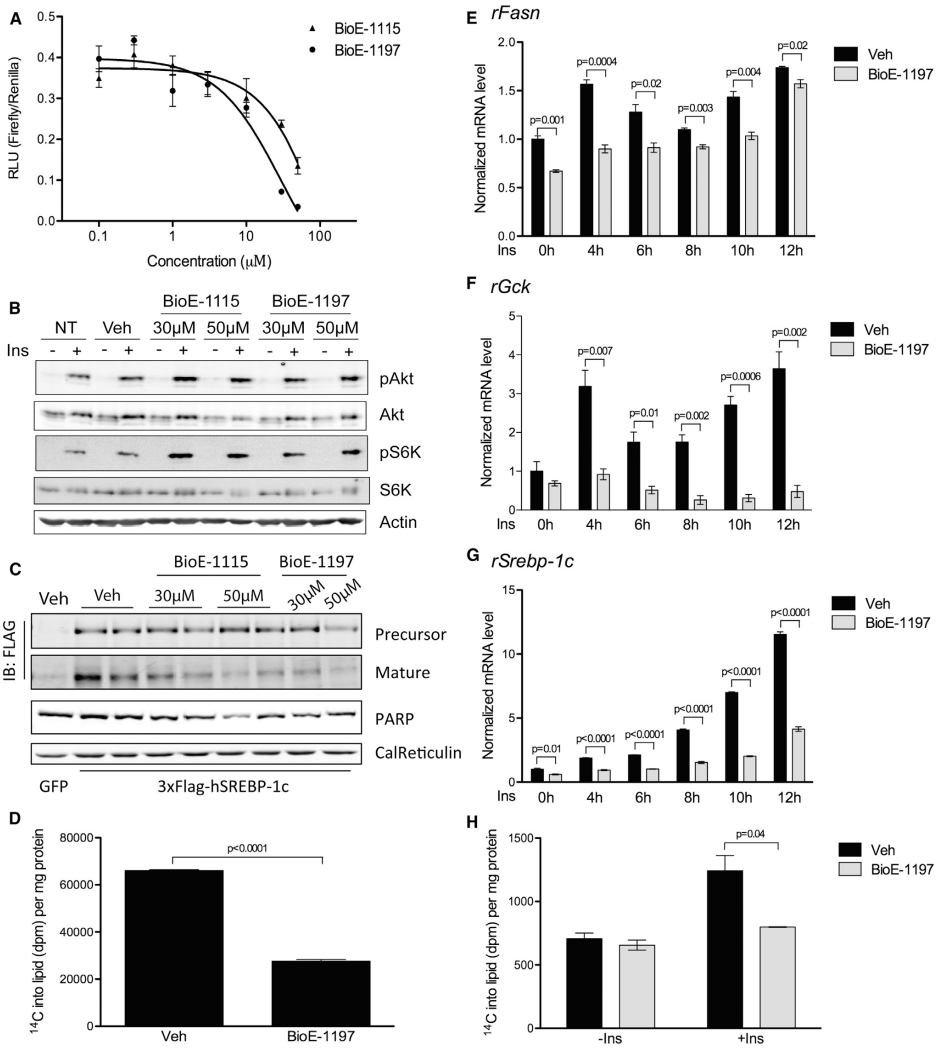

Figure 5. Pharmacological Inhibition of PASK Suppresses SREBP-1 Activation

(A) SRE-Luc activity was measured in HepG2 cells treated overnight with vehicle or the indicated doses of BioE-1115 or BioE-1197 followed by 100 nM insulin for 6 hr. Data are normalized to vehicle-treated samples and shown as the average of n = 3 ± SD.

(B) HepG2 cells were treated as in (A). Cell lysates were subjected to immunoblot to determine the phosphorylation state and abundance of the indicated proteins.

(C) HepG2 cells were infected as in Figure 3A and treated with PASK inhibitors and insulin as in (A). Whole-cell lysates and nuclear extracts were subjected to immunoblot to determine the abundance of indicated proteins.

(D) ^{14}C-acetate incorporation into lipid was measured in BioE-1197-treated HepG2 cells and normalized to the total protein in the lysate. Data shown are the average of n = 3 ± SD.

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Insulin regulates SREBP-1c at almost every level of its expression and stability. Expression of the *SREBF-1* gene is robustly stimulated by insulin (Raghow et al., 2008). The precursor SREBP-1c protein is synthesized in a latent, inactive form that is embedded in the ER membrane. Upon insulin stimulation, SREBP-1c translocates to the Golgi, where the protein encounters two proteases that cleave it to release an N-terminal fragment that is competent to bind DNA and activate gene expression. SREBP-1c maturation is controlled by two associated proteins, SCAP and INSIG (Raghow et al., 2008). Insulin increases the transcriptional activation potential of mature SREBP-1c (Dif et al., 2006; Kotzka et al., 1998). Finally, insulin also impedes the otherwise rapid degradation of the mature, nuclear form of SREBP-1c (Raghow et al., 2008). In total, this multiplicity of regulatory processes enables a robust induction of SREBP-1c by insulin that is both rapid and sustained.

The principal regulatory point at which PASK acts appears to be the proteolytic maturation of the precursor to mature SREBP-1c. This is best evidenced by the decrease in the mature form and increase in the precursor form observed in cells subjected to *PASK* knockdown or inhibition. A specific decrease in the mature form is also observed in *Pask*$^{-/-}$ mice in the fed state. We cannot eliminate the possibility, however, that PASK might also regulate the synthesis or stability of the *SREBF-1* mRNA. We observed that *Pask*$^{-/-}$ liver, *PASK* knockdown HepG2 cells, and primary hepatocytes with PASK inhibition all had lower *SREBP-1c* mRNA levels than the controls. However, this could be explained by the fact that SREBP-1c positively regulates its own gene expression (Amemiya-Kudo et al., 2000; Chen et al., 2004). If the loss of PASK impairs the proteolytic activation of SREBP-1c, this would secondarily lead to a decrease in the *SREBP-1c* mRNA level. In fact, we found that PASK inhibition decreased the activity of a luciferase reporter driven by the *SREBP-1c* promoter in response to insulin, but this effect was completely eliminated when the autoregulatory SREBP-binding site within the promoter was mutated. It remains possible that PASK acts at other steps to regulate SREBP-1c activity; nevertheless, our data do show that normal insulin-responsive maturation requires PASK.

The mechanisms whereby insulin signaling promotes the proteolytic activation of SREBP-1 are still incompletely understood. It has been observed that Akt, in response to insulin signaling, leads to the phosphorylation of SREBP-1c in a manner that correlates with activation (Yellaturu et al., 2009a). Although the functional significance of this SREBP-1c phosphorylation has not been established yet, it is possible that PASK might directly phosphorylate SREBP-1c to promote its maturation. More recent work has shown that insulin signals through both mTORC1-dependent and mTORC1-independent pathways to promote SREBP-1 activation (Wan et al., 2011; Yecies et al., 2011). The latter involves the regulation of INSIG2a, which is a negative regulator of SREBP-1 (Yecies et al., 2011; Yellaturu et al., 2009b). PASK does not appear to act through this mechanism, since *INSIG2* mRNA is not higher upon PASK knockdown, as would be expected if this were the explanation for impaired SREBP-1 processing. In fact, the *INSIG2* mRNA is lower upon PASK knockdown, perhaps as a compensatory effect in response to impaired SREBP-1 activation.

The proteins and mechanisms that connect mTORC1 activation with stimulation of SREBP-1 processing are currently unknown. Our data demonstrate that PASK is not required for Akt or mTORC1 activation in response to insulin signaling, which leads us to conclude that PASK acts either downstream of mTORC1 or in a parallel pathway to promote SREBP-1 activation. The precise placement of PASK in this signaling network and definition of the mechanisms that connect the nodes of this network await further studies.

Not only did administration of the PASK inhibitor cause profound decreases in the hepatic expression of SREBP-1c target genes and the generation of mature SREBP-1, it also impacted hepatic and systemic metabolic parameters. Both hepatic and serum triglycerides were normalized, which might be expected based on the suppression of lipogenic gene expression in the liver. In addition, we observed a significant decrease in serum glucose in HFrD-fed rats treated with the PASK inhibitor for 7 days, and in HbA1c in rats treated for either 45 or 90 days. In conjunction with the modest decrease in serum insulin, this is suggestive of enhanced insulin sensitivity in inhibitor-treated animals.

A pathological vicious cycle has been described wherein the hypoglycemic effects of insulin are blunted, but insulin maintains the ability to activate SREBP-1c and lipogenesis (Biddinger et al., 2008; Brown and Goldstein, 2008). Enhanced lipogenesis and the increasingly dyslipidemic state exacerbate the ''selective insulin resistance,'' causing more insulin release. We hypothesize that PASK inhibition prevents this vicious cycle by mitigating the toxic lipogenesis that can occur under conditions of insulin resistance and hyperinsulinemia. We previously showed that *Pask*$^{-/-}$ mice exhibit improved insulin sensitivity and resistance to hepatic steatosis elicited by an HFD (Hao et al., 2007). We now also show that treatment of HFrD-fed rats with a PASK inhibitor completely normalized their elevated expression of SREBP-1c target genes and hypertriglyceridemia.

Importantly, we observed no overt toxicity upon genetic depletion of PASK (*Pask*$^{-/-}$ mice) or upon extended treatment with PASK inhibitor doses that were 10- to 30-fold higher than those required to elicit significant effects on lipogenic gene expression and serum triglycerides. This treatment regimen had no effect on body weight, even after 90 days of treatment. It also had no effect on the expression of *SREBP-1c* or its target genes in two other tissues of metabolic importance, skeletal muscle, and adipose tissue. In contrast, inhibition of mTORC1 with rapamycin caused decreased lipogenesis in adipose tissue, which was accompanied by decreased expression of SREBP-1 (Pereira et al., 2013). The selectivity of the effect of PASK inhibition is also in contrast to the phenotype observed for the majority of mice lacking *SREBP-1*, which die in utero (Shimano et al.,

(E–G) Rat primary hepatocytes were treated with vehicle or BioE-1197 as in (D), followed by insulin as in Figure 1I, and the mRNA abundance of the indicated genes was measured as in Figure 1I. Data shown are the average of n = 3 ± SEM. The value of the vehicle-treated/unstimulated group was set as one.
(H) Rat primary hepatocytes were treated and measured for ^{14}C-acetate incorporation into lipids as in (D). Data shown are the average of n = 3 ± SD.
See also Figure S3.

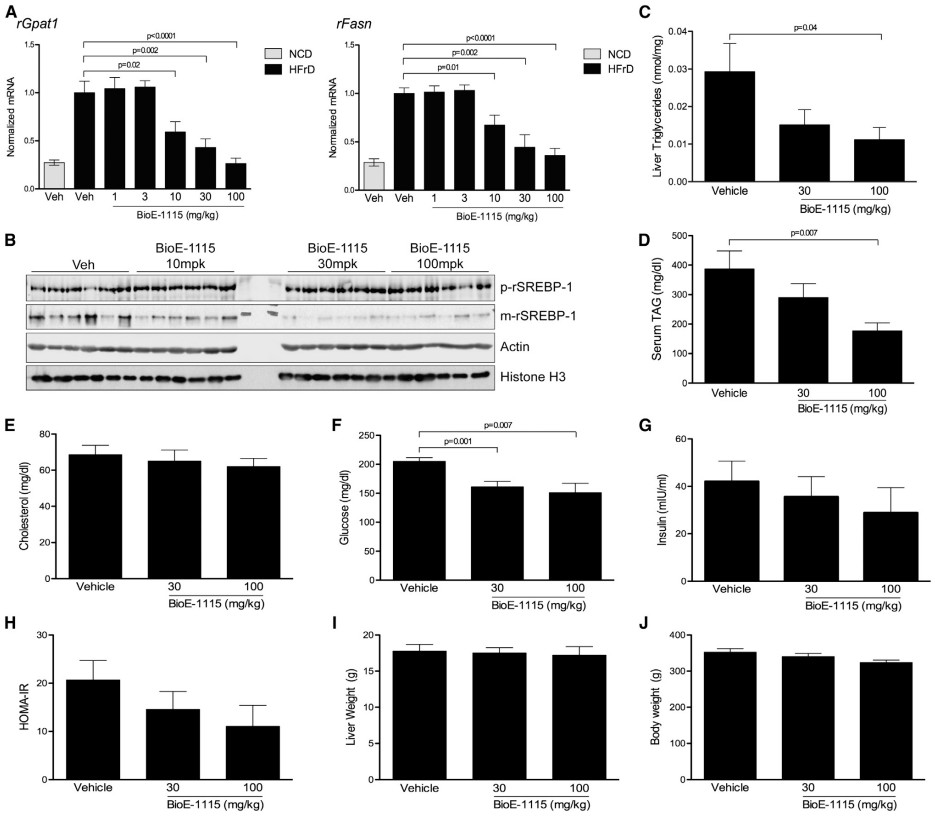

Figure 6. PASK Inhibition Decreases SREBP-1 Activity, Triglycerides, and Insulin Resistance in Animal Models

(A) Rats fed either an NCD or HFrD for 2 weeks were subjected to a once/day treatment with vehicle or the indicated dose of BioE-1115 by oral gavage for 1 week. Following this regimen, the rats were fasted for 24 hr and then refed for 12 hr. Livers were harvested and qRT-PCR was performed for the indicated genes and normalized to *Cyclophilin A* (n = 10/group). Data shown are the average ± SEM.

(B) Rats were maintained on an HF-HFrD for 18 weeks and treated with vehicle or the indicated dose of BioE-1115 as in (A) for the last 3 weeks. Livers were harvested after rats were subjected to fast/refeeding as in (A). Whole-liver lysates and nuclear extracts were subjected to immunoblot to determine the abundance of the precursor or mature form of SREBP-1 and the indicated control proteins. mpk: mg/kg.

(C) Triglycerides were measured in livers from the indicated treatment groups as described in (A) (n = 10/group).

(D–G) Serum TAG, cholesterol, glucose, and insulin were measured in animals treated as in (A).

(H) Calculated HOMA-IR values.

(I and J) Liver (I) and body (J) weights of the animals in (A). All data shown are the average ± SEM. See also Figures S4–S6.

1997b), whereas mice lacking *PASK* are viable. Therefore, we conclude that PASK is not required for the basal activation of SREBP-1 or for the activation of SREBP-1 in all tissues. Our data suggest an important role for PASK in the hepatic activation of SREBP-1c in response to feeding. However, it is possible that PASK has functions in other tissue types that may contribute to the beneficial effect on metabolism we observed upon PASK inhibition. Nonetheless, this observation is of particular interest in light of the potentially causal role that hepatic SREBP-1c activation has been proposed to play in metabolic disease in humans. Others have shown that genetic or pharmacological inhibition of SREBP maturation improves hepatic and whole-body metabolism (Moon et al., 2012; Tang et al., 2011). We therefore propose that SREBP-1c activation by PASK is an important

feature of the mammalian metabolic syndrome and should be explored as a therapeutic opportunity in humans.

EXPERIMENTAL PROCEDURES

Animals

Pask$^{-/-}$ mice were described previously (Hao et al., 2007). Age-matched male WT and *Pask*$^{-/-}$ mice were maintained on either an NCD for 12 weeks or a 60% HFD for 8 weeks, from 8 to 10 weeks of age (60% fat by calories; Research Diets). For fasting-refeeding studies, mice were either fasted for 24 hr or fasted for 24 hr and refed an NCD or HFD for the indicated time periods before they were euthanized and their organs were harvested. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

SD male rats (Charles River Laboratories) were maintained on an HFrD (60% fructose; Research Diets) or NCD for 2 weeks or on a high-fat and high-fructose diet (HF-HFrD, 60% high fat diet and 15% fructose drinking water) for 15 weeks before experiments were conducted. The rats were housed under standard vivarium conditions (12 hr light/dark cycle) with water and chow ad libitum. All studies were approved by the Institutional Animal Care and Use Committee of the University of Utah and/or St. Louis University as appropriate.

Luciferase Assay

HepG2 cells were cotransfected with (1) pGPAT-Luc, pSCD-Luc, or pSRE-Luc; (2) a construct expressing CMV-driven Renilla luciferase (Promega); or (3) a pQCXIN-GFP, pcDNA3.1-2xFlag-mSREBP-1a, or pQCXIN-3xFlag-pSREBP-1a construct, as indicated, using Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions. Rat primary hepatocytes were cotransfected with WT or mutant pSREBP-1c-Luc and Renilla luciferase using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were serum-starved overnight, with additional treatment of (1) vehicle, BioE-1197, or BioE-1428 and/or (2) 100 nM insulin for 6 or 12 hr before harvest, as indicated. Firefly and Renilla luciferase were assayed using the Dual-Reporter Luciferase Assay System (Promega).

Measurement of De Novo Lipogenesis

HepG2 cells or rat primary hepatocytes were treated with vehicle or 10 μM BioE-1197 in serum-free media overnight. The next day, the cells were transferred to new media with vehicle or 30 μM BioE-1197 and 100 nM insulin for 6 hr as indicated, and were labeled with 10 μCi/ml [1-^{14}C]-acetate (Perkin Elmer) for the last 4 hr before harvest. The cells were washed twice with PBS and then lysed in 0.5% Triton X-100. The lipid fraction was extracted by adding chloroform and methanol (v/v 2:1) followed by dH_2O, with vortexing. Samples were then centrifuged at 1,500 rpm for 15 min and the organic (bottom) phase containing lipids was used to measure ^{14}C incorporation on a Beckman LS 6500 scintillation counter. The results were normalized to protein concentration of lysates.

ELISA

HEK293T cells were transfected with a pcDNA3.1-Flag-PASK (WT or kinase-dead) construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 18 hr, the cells were reseeded into 96-well plates in Dulbecco's modified Eagle's medium/1% fetal bovine serum. The cells were then treated with DMSO or drug for 16 hr, followed by lysis in 0.2 ml lysis buffer (20 mM Na_2HPO_4, 0.5% Triton, 0.1% SDS, 0.02% azide, 1 mM NaF, 1 mM glycerophosphate, 1 mM Na_3VO_4). The lysates were then applied to a MaxiSorb 96-well plate (Nunc) that was previously coated with α-FLAG capture antibody (M2; Sigma), and blocked with 3% BSA (in 1× PBS). After incubation at 4°C for 1.5 hr, the plates were washed with high-salt washing buffer (20 mM Na_2HPO_4, 0.5% Triton X-100, 0.1% SDS, 0.02% NaN_3, 0.1% BSA, and 1 M NaCl) followed by low-salt buffer (150 mM NaCl). Then the plates were incubated for 2 hr with antibody to either phospho-Akt substrate (9614; Cell Signaling) or hPASK (U2501), followed by high- and low-salt buffer washes. Subsequently, the plates were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr and then washed with high- and low-salt buffer and 1× PBS. The phospho-AKT substrate or PASK antibody-dependent luminescence signal was assayed using the LumiGLO chemiluminescent substrate system (KPL) according to the manufacturer's instructions. The inhibition curve and IC_{50} were determined using Prism software (GraphPad).

Chemicals and Dosing Formulations

BioE-1197, BioE-1428, and BioE-1115 were synthesized by Pharmaron. All compounds were made up in a vehicle formulation of 0.5% methylcellulose and 0.025% Tween-80 (Sigma) in ddH_20. Dosages were calculated and compounds were weighed and placed in a 15 ml glass homogenizer (Kimble Chase) to which vehicle was added. The compounds were ground to a fine suspension and transferred to a screw-top tube. The homogenizer was rinsed twice with vehicle and then brought to the final volume. Compounds were made up every 4–5 days based on the animals' weight and stored at room temperature. The animals were orally dosed once a day between 7 a.m. and 9 a.m.

BioE-1115 in the SD Diet-Induced Obesity Model

Male SD rats (n = 8–12) were obtained in this study with an average weight of 129.4 ± 0.63 g and were maintained on an HFrD for 2 weeks or on an HF-HFrD for 15 weeks prior to experimentation. The rats were dosed by oral gavage once a day for 7, 21, or 90 days at doses of 1, 3, 10, 30, and 100 mg/kg of BioE-1115 or with vehicle. Body weights were taken every day for compound formulation. The animals were fasted for 24 hr and refed for 12 hr prior to termination of the experiment. All animals received their respective compound dosage 3 hr prior to termination. The animals were euthanized by CO_2 asphyxiation, and cardiac puncture was performed for the final serum analysis. Liver tissue was taken during necropsy, weighed, and snap-frozen in liquid N_2. Blood was centrifuged at 3,500 rpm for 10 min and serum was collected and analyzed for insulin, glucose, cholesterol, and triglycerides using a Beckman CX 5Pro (Beckman Coulter).

Statistical Analysis

Data are presented as mean ± SD unless otherwise indicated. A two-tailed equal variance t test was used to compare differences and the null hypothesis was rejected at the 0.05 level.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.006.

AUTHOR CONTRIBUTIONS

X.W., W.I.S., I.D., C.K.K., H.S., B.S.Z., and G.A.N. designed and conducted experiments. D.R. led the medicinal chemistry program. X.W., J.M., J.T.B., G.A.N., and J.R. designed experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTAL FIGURES and LEGENDS

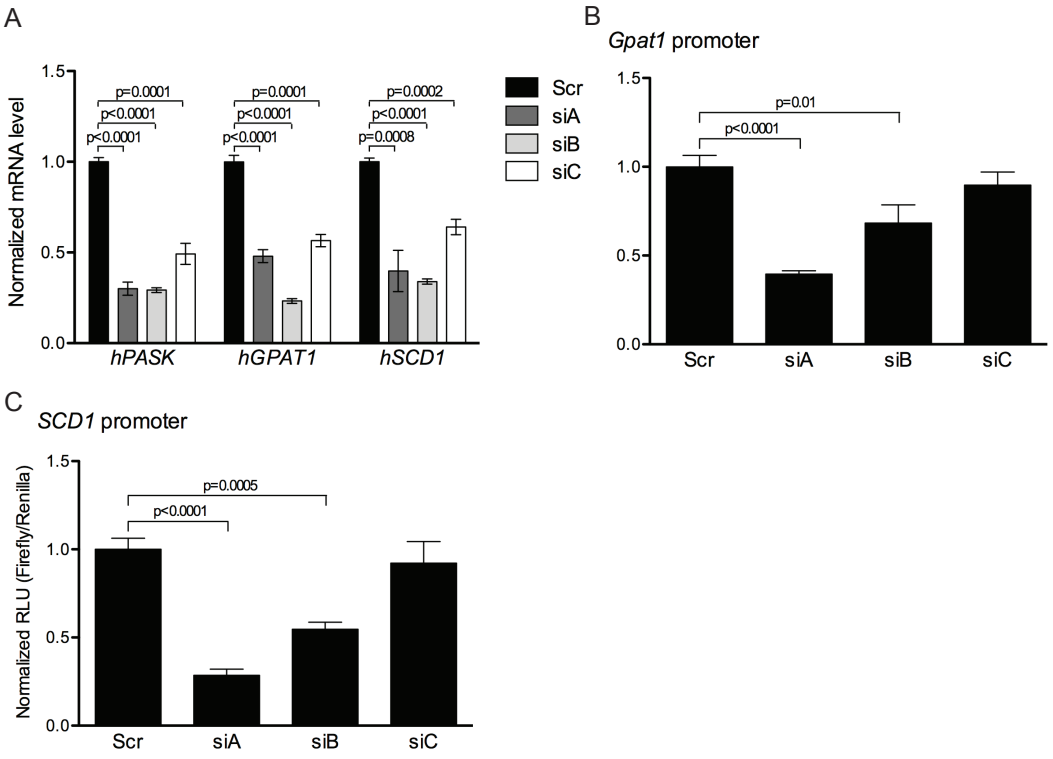

Figure S1. PASK depletion leads to lower SREBP-1 activity in HepG2 cells, related to Figure 2. (A) HepG2 cells were treated with scrambled or *PASK*-specific siRNA (siA, siB or siC). Cells were serum starved overnight, and mRNA levels of the indicated genes were measured by qRT-PCR and normalized to *Tubulin* mRNA levels. Data shown are the average of n=3 ± SEM, with the Scr value set as 1. (B and C) *PASK* silenced HepG2 cells were transfected with Gpat1-Luc (B) or SCD1-Luc (C) as well as Renilla luciferase (as a normalizing control). Cells were then serum starved overnight, and firefly and Renilla luciferase were assayed using the Dual-Reporter Luciferase Assay System. Data shown are the average of n=3 ± SD, with the scr value set as 1.

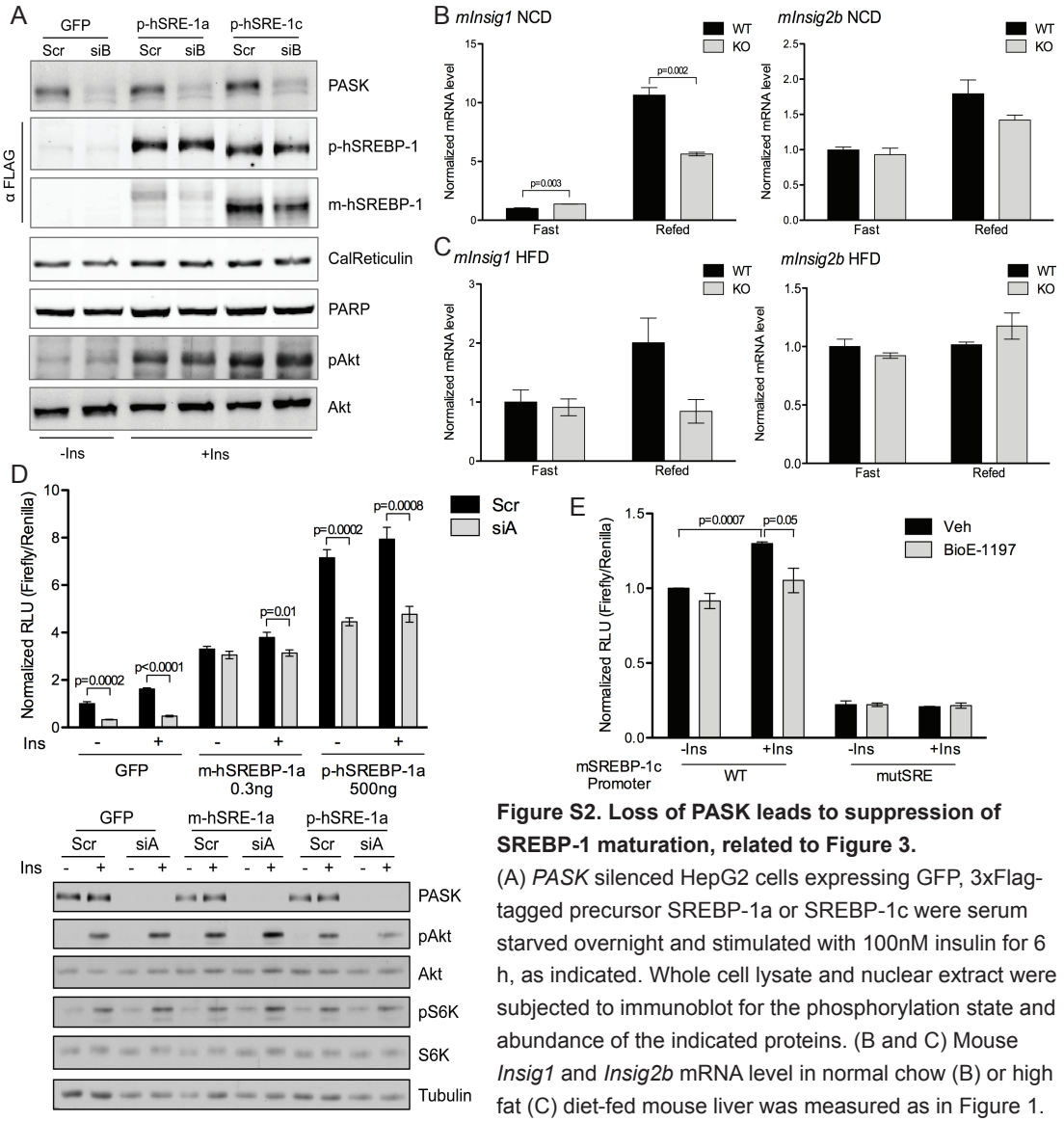

Figure S2. Loss of PASK leads to suppression of SREBP-1 maturation, related to Figure 3.

(A) *PASK* silenced HepG2 cells expressing GFP, 3xFlag-tagged precursor SREBP-1a or SREBP-1c were serum starved overnight and stimulated with 100nM insulin for 6 h, as indicated. Whole cell lysate and nuclear extract were subjected to immunoblot for the phosphorylation state and abundance of the indicated proteins. (B and C) Mouse *Insig1* and *Insig2b* mRNA level in normal chow (B) or high fat (C) diet-fed mouse liver was measured as in Figure 1. Data shown are the average of n=3 ± SEM, with the "WT fasted" value set as 1. (D) Top: SRE-Luc activity was measured in *PASK* silenced HepG2 cells co-transfected with a construct expressing GFP or mature or precursor SREBP-1a along with SRE-Luc. Data shown are the average of n=3 ± SD, with the scr value set as 1. Bottom: Following luciferase assay, cell lysates were subjected to immunoblot for the phosphorylation state and abundance of the indicated proteins. (E) Rat primary hepatocytes were transfected with plasmids expressing luciferase driven by either wild-type mouse *SREBP-1c* promoter, or a mutant promoter with disrupted SRE. Cells were then treated with BioE-1197 and 100nM insulin as indicated. Firefly and Renilla luciferase were assayed. Duplicate plates were prepared for each condition, and each plate was assayed in triplicate. Data are show as average ± SD.

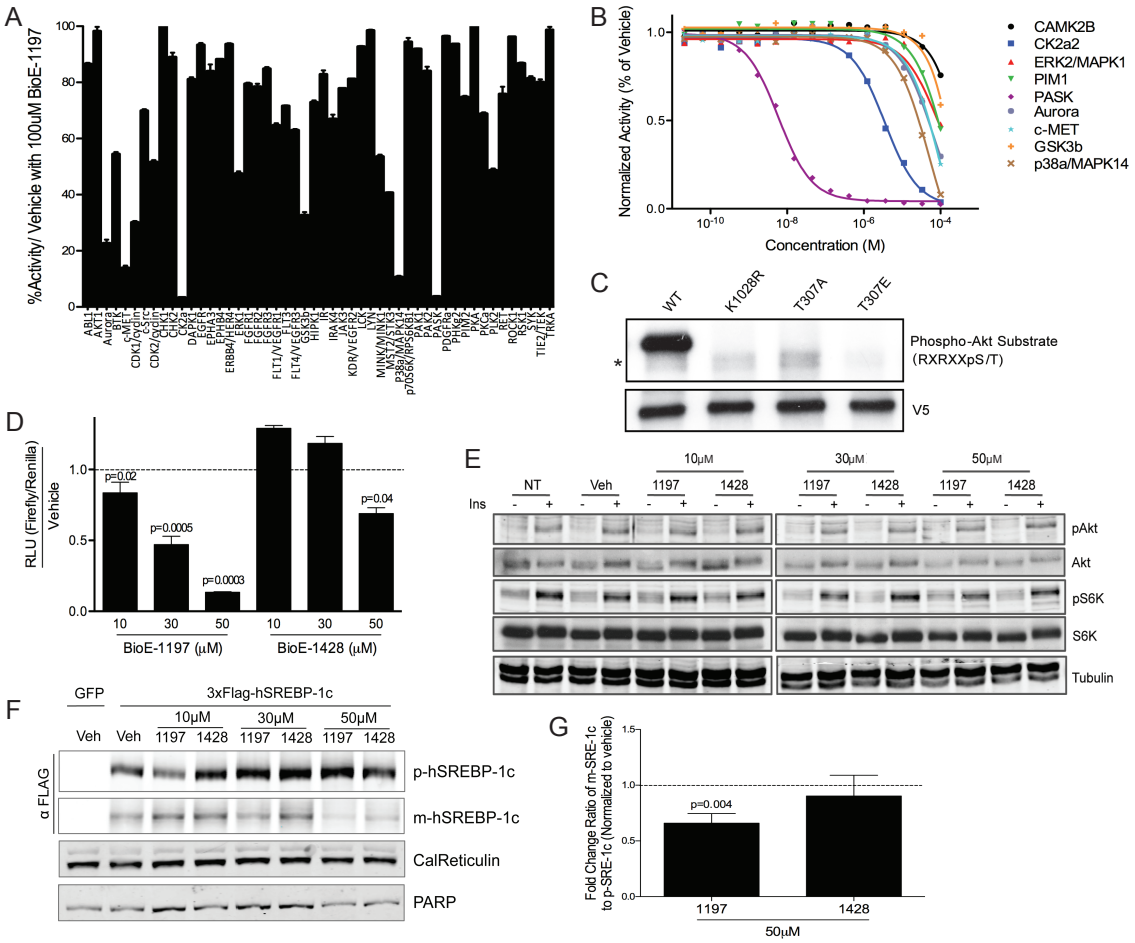

Figure S3. Pharmacological inhibition of PASK leads to lower SREBP-1 activity, related to Figure 4 and 5. (A) Activity of the indicated kinases was measured in the presence of vehicle or 100μM BioE-1197 and the percent activity in the presence of drug is indicated. (B) The indicated kinases were assayed in the presence of the indicated concentrations of BioE-1197 and the percent of vehicle-treated activity is indicated. (C) PASK was immunoprecipitated from HEK293 cells expressing WT, kinase-dead (K1028R), T307A or T307E mutant V5-tagged hPASK using anti-V5 antibody, and subjected to immunoblot for the phosphorylation state and abundance of the indicated proteins. The asterisk (*) indicates nonspecific bands.(D) HepG2 cells were transfected with SRE-Luc and Renilla luciferase, treated with vehicle, or indicated doses of BioE-1197 or BioE-1428 overnight, and stimulated with 100nM insulin for 6h. Firefly and Renilla luciferase were assayed and the ratio was normalized to vehicle treated samples and shown as the average of n=3 ± SD. (E) HepG2 cells were either not treated (NT), or treated with vehicle or PASK inhibitors and insulin as in (D). Cell lysates were subjected to immunoblot for the phosphorylation state and abundance of the indicated proteins. (F) HepG2 cells were infected as in Figure 5C. Cells were then treated with PASK inhibitors and insulin as in (D). Whole cell lysate and nuclear extract were subjected to immunoblot for the phosphorylation state and abundance of the indicated proteins. (G) The ratio of precursor to mature form of SREBP-1c was quantified for both compounds at 50μM concentration and normalized to vehicle treated cells. Data shown are the average of n=5 ± SEM.

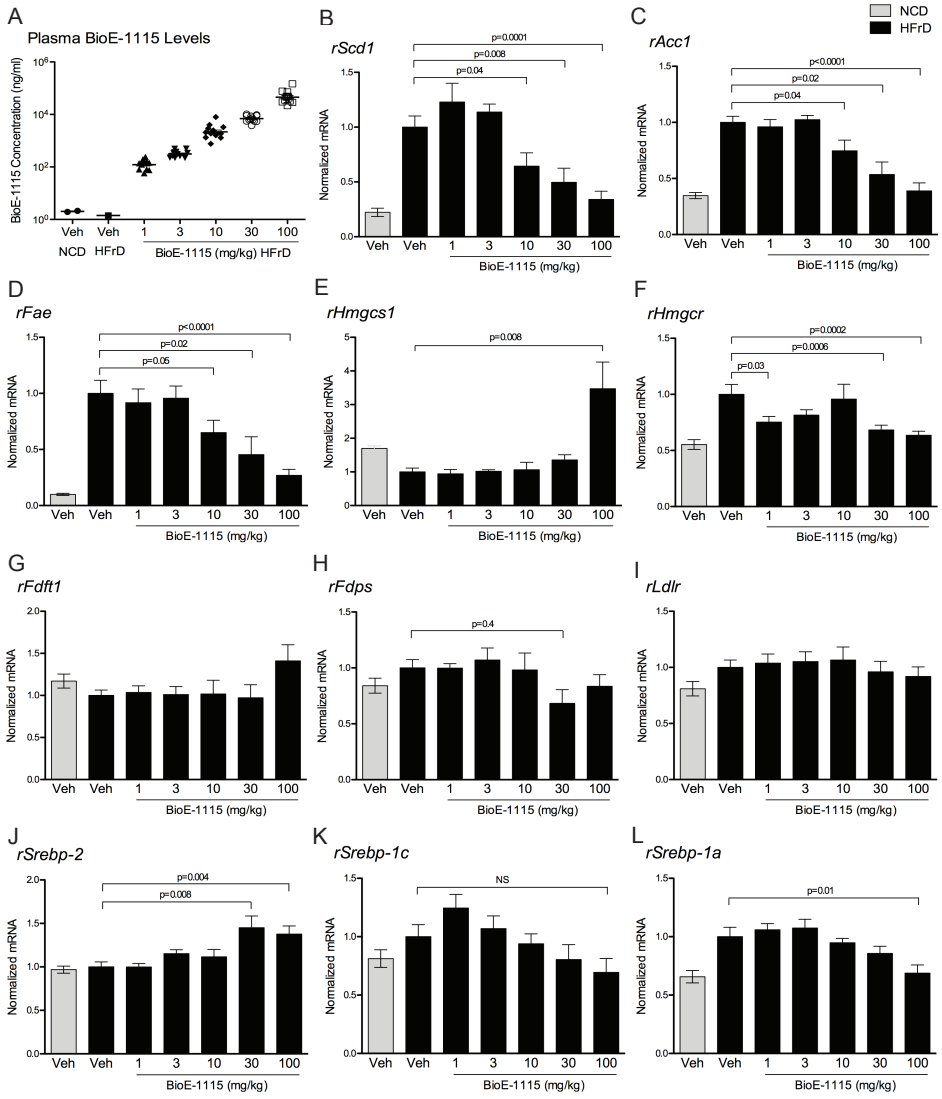

Figure S4. Compound and transcripts levels in vehicle or BioE-1115 treated rat livers, related to Figure 6.

(A) Rats fed either normal chow or high-fructose diet for 2 weeks were subjected to once/day treatment with vehicle or the indicated dose of BioE-1115 by oral gavage for one week. Following this regimen, rats were fasted for 24 h and then refed for 12 h. Plasma BioE-1115 levels were measured (n=10/group). (B-L) Livers were harvested from animals in (A) and qRT-PCR was performed as described in Figure 6A. All data shown are the average ± SEM. NS, not significant.

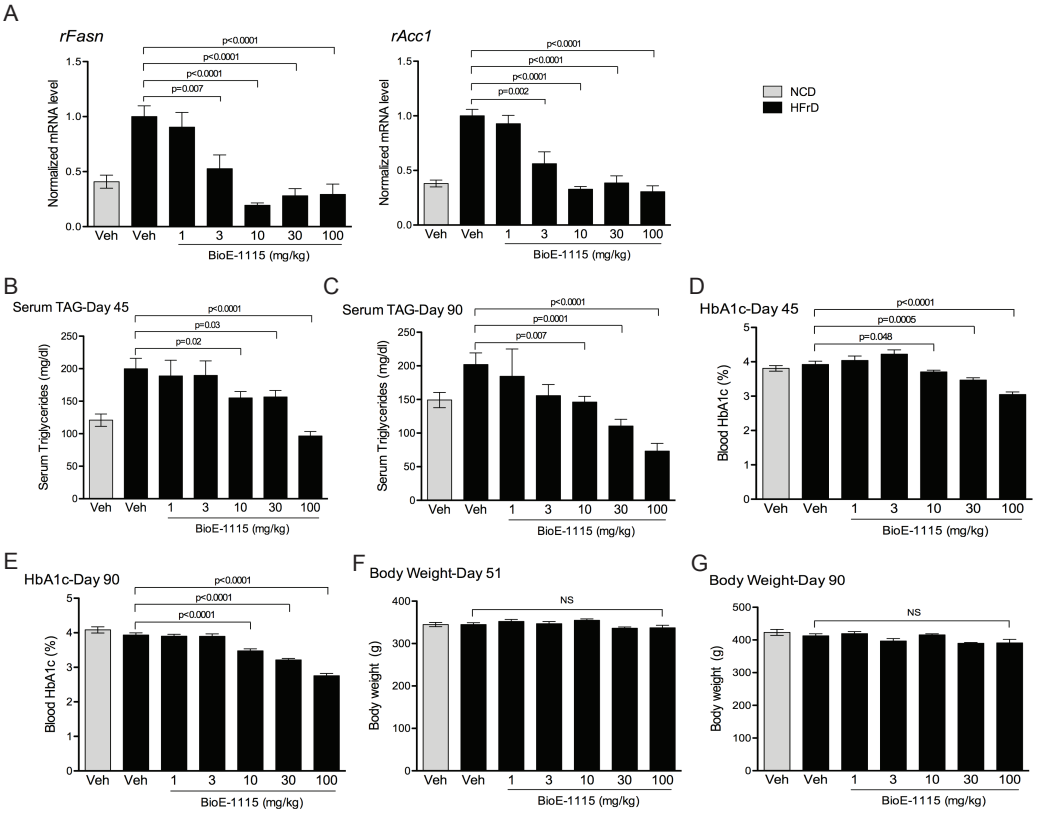

Figure S5. Effect of long-term PASK inhibition in animal models, related to Figure 6.
(A) Rats were treated and analyzed as in Figure 6A, except they were subjected to once/day treatment with vehicle or the indicated dose of BioE-1115 by oral gavage for 90 days. Livers were harvested and qRT-PCR was performed for the indicated genes and normalized to *Cyclophilin A* (n≥12/group). (B-E) Serum TAG and glycated hemoglobin (HbA1c) were measured in the animals from (A) at either 45 days or 90 days of administration. (F and G) Body weight was measured after indicated length of treatment (n≥12/group). All data shown are the average ± SEM. NS, not significant.

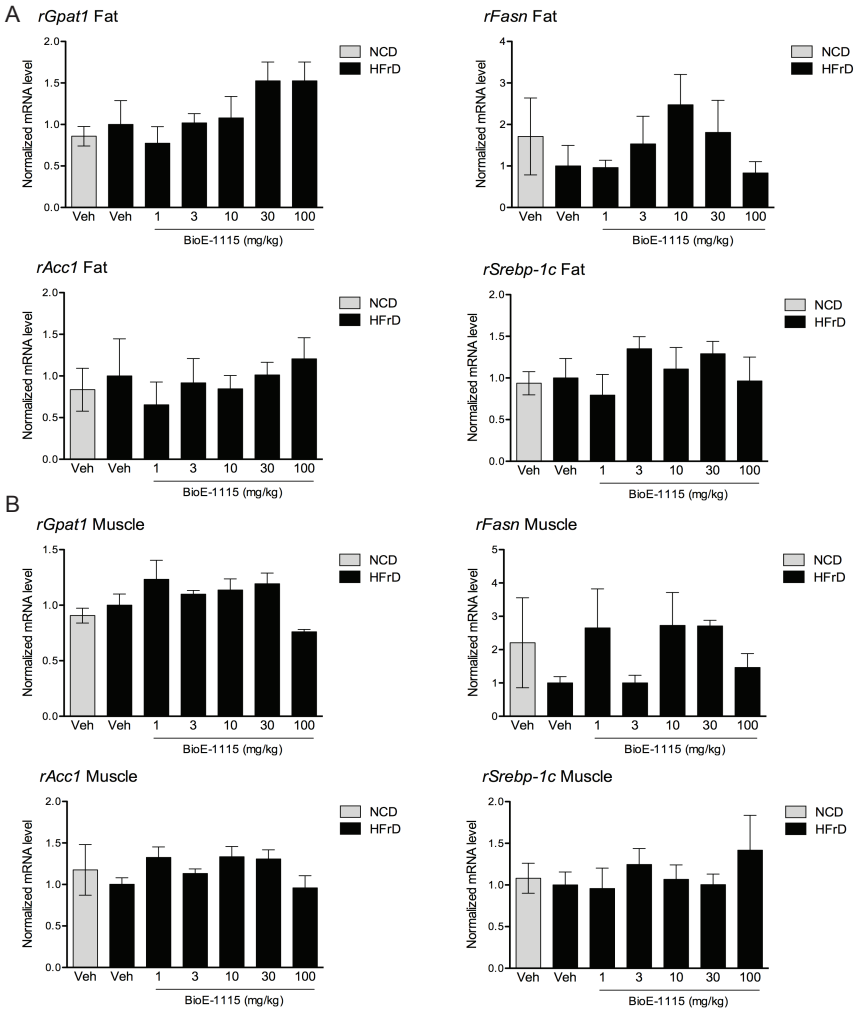

Figure S6. mRNA levels of various genes in vehicle or BioE-1115 treated rat fat and muscle, related to Figure 6.

Rats fed either normal chow or high-fructose diet for 2 weeks were subjected to once/day treatment with vehicle or the indicated dose of BioE-1115 by oral gavage for 90 days. Following this regimen, rats were fasted for 24 h and then refed for 12 h. Abdominal fat (A) and gastrocnemius muscle (B) were harvested and qRT-PCR was performed as described in Figure 6A (n≥12/group). All data shown are the average ± SEM.

SUPPLEMENTAL TABLES

Table S1. Hepatic mRNA levels of various genes in HFD-fed wild-type and *Pask*$^{-/-}$ mice.

Gene	Fast		Refed	
	WT	KO	WT	KO
mSrebp-1a	0.89±0.19	0.70±0.12	1.40±0.08	1.10±0.08*
mSrebp-1c	0.70±0.20	0.42±0.09	1.53±0.15	0.90±0.14*
mSrebp-2	0.83±0.14	0.91±0.12	1.17±0.11	1.17±0.06
mHmgcs1	0.56±0.07	0.94±0.31	1.65±0.30	1.77±0.35
mHmgcr	0.45±0.04	0.74±0.09**	1.34±0.15	1.18±0.17

Wild-type and *Pask*$^{-/-}$ mice on the C57/BL6J background were maintained on a 60% high-fat diet (HFD) for 8 weeks (n≥4/group). Before harvesting, mice were fasted for 24 h, or fasted for 24 h and refed a 60% HFD for 12 h. Livers were harvested and mRNA levels of indicated genes were measured by qRT-PCR and normalized to *Cyclophilin A* mRNA levels. Data shown are the average ± SEM, with the “WT fasted” value set as 1. *p<0.05; **p<0.01.

Table S2. Hepatic mRNA levels of various genes in NCD-fed wild-type and *Pask*$^{-/-}$ mice.

Gene	Fast		Refed					
			2h		6h		8h	
	WT	KO	WT	KO	WT	KO	WT	KO
mSrebp-1a	0.55±0.09	0.58±0.01	0.97±0.09	0.96±0.04	1.16±0.22	0.86±0.02	1.14±0.15	0.82±0.04
mSrebp-1c	0.26±0.01	0.27±0.02	0.70±0.24	0.72±0.23	0.93±0.27	1.19±0.14	2.51±0.88	1.49±0.26
mSrebp-2	0.46±0.04	0.50±0.17	0.77±0.14	0.62±0.05	1.16±0.10	0.85±0.04*	1.00±0.11	0.78±0.08
mHmgcs1	0.36±0.09	0.22±0.03	0.53±0.24	0.36±0.03	1.89±0.16	1.58±0.32	2.46±0.28	1.98±0.25
mHmgcr	0.18±0.03	0.14±0.02	0.73±0.27	0.46±0.05	3.03±0.34	1.50±0.27*	2.56±0.25	1.26±0.02**

Wild-type and *Pask*$^{-/-}$ mice on the C57/BL6J background were maintained on a normal chow diet (NCD) for 12 weeks. Before harvesting, mice were fasted for 24 h, or fasted for 24 h and refed an NCD for indicated time periods. Livers were harvested and mRNA levels of indicated genes were measured by qRT-PCR and normalized to *Cyclophilin A* mRNA levels. Data shown are the average of n=3 ± SEM, with the “WT fasted” value set as 1. *p<0.05; **p<0.01.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture

HepG2 and HEK293T cells were obtained from ATCC. HepG2 cells were maintained in 1:1 DMEM/F-12 (HyClone) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma). HEK293T cells were maintained in DMEM (Mediatech) supplemented with 10% FBS and 1% penicillin/streptomycin.

Rat Primary Hepatocytes

Primary hepatocytes were isolated from non-fast 3-month-old male Sprague-Dawley (SD) rats. The liver was perfused through portal vein with Liberase (Roche), and hepatocytes were isolated using percoll (Sigma) gradient purification. Isolated hepatocytes were then plated onto rat-tail collagen type I (Roche)-coated plates in DMEM (Mediatech) containing 10% FBS and incubated for 3-5 h at 37°C. Cells were then washed twice with PBS, incubated in Medium 199 (Invitrogen) supplemented with 100nM Dexamethasone (Sigma) and 1% penicillin/streptomycin, and subjected to additional treatments including plasmid transfection, PASK inhibitor and/or insulin, as indicated.

Gene Expression Analysis

Total RNA was isolated from tissues or cells using RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. cDNA synthesis was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR

Green-based real-time PCR was performed using a LightCycler 480 Real-Time PCR System (Roche). Primer sequences for indicated transcripts are available on request.

PASK Silencing

HepG2 cells were transfected with scrambled (Allstars negative control siRNA, Qiagen) or human *PASK*-specific siRNAs (Qiagen) using HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. Cells were analyzed 96 h after transfection.

Plasmids and Plasmid Constructions

pGPAT-Luc and pSCD-Luc were generated by cloning the promoter region of mouse *Gpat1* (Yoshida et al., 2009) and human *SCD1* (Bene et al., 2001), respectively, followed by ligation into PGL-3 vector. For expression of the precursor form of SREBP-1a or SREBP-1c, the N-terminally Flag-tagged SREBP-1a or SREBP-1c was cloned from human SREBP-1 cDNA (BC057388; Open Biosystems) and ligated into the pQCXIN retroviral vector to generate pQCXIN-3xFlag-pSREBP-1a or -1c. Wild-type and kinase-dead (K1028R) human PASK were cloned into a V5 or Flag-tagged mammalian expression vector (pcDNA3.1-V5/His, pcDNA3.1-Flag), as described previously (Kikani et al., 2010). T307A and T307E mutant hPASK were generated from wild-type construct using a QuickChange site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing. pSRE-Luc, a plasmid containing the hamster HMG-CoA synthase sterol regulatory elements fused to luciferase cDNA, pcDNA3.1-2xFlag-mSREBP-1a, a plasmid expressing the mature form of human SREBP-1a, and pSREBP-1c-Luc, a plasmid containing 900bp

fragment of the mouse *Srebp-1c* promoter fused to luciferase cDNA, were kind gifts of Dr. Timothy F. Osborne (Sanford-Burnham Medical Research Institute). Mutant pSREBP-1c-Luc with disrupted SRE element was generated using the following primers:
CGCGGCTGCTGATTGGCCACTGCAGTCTCGCTGAGGGGCGGGGC (F) and
CGATCGCCTCCGTGCCCCGCCCCTCAGCGAGACTGCAGTGGCCAATC (R).
pQCXIN-GFP was a kind gift of Dr. Wesley I. Sundquist (University of Utah).

Retroviral Production and Infection

To generate virus, pQCXIN-GFP, pQCXIN-3xFlag-pSREBP-1a or -1c construct was cotransfected with Gag-pol envelope and VSV-G packaging plasmids into HEK293T cells using Lipofectamine 2000 (Invitrogen), according to the manufacture's instructions. 48 h after transfection, viral supernatant was collected and centrifuged to remove cellular debris. HepG2 cells were infected with viral supernatant overnight in the presence of 8μg/ml polybrene. Cells were analyzed 24-48 h after infection.

Nuclear Extract Preparation

HepG2 cells were serum-starved overnight, with additional treatment of (1) vehicle, BioE-1197 or BioE-1428, and/or (2) 100nM insulin for 6 h before harvest, as indicated. 1 h before harvest, cells were treated with 25μg/ml ALLN (Calbiochem). To prepare nuclear extract, HepG2 cells were washed, collected by scraping and pelleted in hypotonic buffer (10mM HEPES, PH 7.9, 0.1mM EDTA, 0.1mMEGTA,

10mM KCL, 1mM DTT, 0.5mM PMSF and 1X mammalian protease inhibitor cocktail [Sigma]). Cells were then resuspended in the same buffer, and a small aliquot was taken and saved as whole cell lysate. The remaining cells were incubated on ice for 30 min. Plasma membranes were disrupted by adding 0.1% NP-40 and passing through a 25 gauge syringe needle 15 times. The nuclei were pelleted by centrifugation at 6000 rpm for 1 min at 4°C, washed with hypotonic buffer, and pelleted again by centrifugation at 10,000 rpm for 2 min at 4°C. The purified nuclei were then dissolved in 1X SDS sample buffer and subjected to immunoblot.

To extract nuclear protein from mouse liver, fresh tissue was harvested and homogenized in ice-cold PBS, followed by centrifugation at 900xg for 5 min at 4°C. Pellet was then resuspended in Homogenization buffer (10mM HEPES, pH 7.6, 25mM KCl, 2M sucrose, 10% glycerol, 0.15mM spermine, 1.0mM PMSF) and homogenized on ice with a Dounce tissue grinder. Nuclei were pelleted by ultracentrifugation at 26K rpm for 1h at 4°C, and resuspended in nuclear lysis buffer (50mM Tris, pH 7.6, 0.5M NaCl, 1.0mM PMSF). Samples were then incubated on ice for 30 min followed by sonication and centrifugation at 14,000 rpm for 20 min at 4°C. Supernatant was collected as nuclear extract and subjected to immunoblot. All the buffers mentioned above contain 1X mammalian protease inhibitor cocktail (Sigma).

Immunoprecipitation and Immunoblot

V5-tagged wild-type and various mutant forms of human PASK were expressed and immunoprecipitated from HEK293T cells, as described previously (Kikani et al., 2010). For immunoblot, HepG2 cell lysates were prepared by cell fractionation or by directly dissolving cells in 1X SDS sample buffer. Protein samples were then separated by SDS-PAGE and transferred to nitrocellulose for blocking and incubation with primary antibody and secondary antibody conjugated with either HRP for detection by ECL/ECL Plus kit (PerkinElmer), or infrared dyes for detection by the Odyssey system (LI-COR). Quantitation of protein bands was performed using the built-in Odyssey software. Primary antibodies were obtained from the following sources: anti-hPASK (U2501) made in the lab; anti-Flag (M2) and anti-Actin (A5441) from Sigma; anti-SREBP-1 (557036) from BD Bioscience; anti-V5 from Invitrogen; anti-Histone H3 (1791) from Abcam; antibodies to Tubulin (2148), PARP (9542), CalReticulin (2891), Akt (9272), S6K (9202), phospho-T308 Akt (9275), phospho-T389 S6K (9205) and phospho-Akt substrate (9614) from Cell Signaling.

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CHAPTER 3

CONCLUDING REMARKS

In this dissertation, we have identified PASK as a novel regulator of SREBP-1c, the master transcription factor that drives the lipogenic program in liver in response to feeding and insulin signaling. Using genetic and pharmacological approaches, we showed that PASK is required for the proteolytic maturation of SREBP-1c in response to feeding and insulin stimulation. Genetic depletion and pharmacological inhibition of PASK result in lower levels of mature SREBP-1, decreased expression of its lipogenic target genes and reduced lipid production in cultured cells and in rodent livers. Interestingly, the expression of PASK itself is also upregulated in response to feeding and insulin, consistent with its function in promoting lipogenesis.

As the master transcriptional regulator of the lipogenic program, hyperactivation of SREBP-1c is causally linked to hepatic steatosis (NAFLD), hypertriglyceridemia and other metabolic disorders. Metabolic diseases have become a new epidemic around world. Failure to properly maintain energy balance at the whole-body and cellular levels is at the heart of these diseases. Cellular energy balance is maintained via two well-characterized energy and nutrient sensors, AMP-activated protein kinase (AMPK) and mechanistic Target of Rapamycin (mTOR). AMPK senses low cellular energy levels and suppresses biosynthetic processes, whereas mTOR responds to nutrient abundance and stimulates biosynthetic processes. Thus, together, these two protein kinases function to coordinate cellular metabolism with nutrient environment and energy status. Dysreguation of AMPK and mTOR signaling pathways has been shown to play vital roles in the development of metabolic disorders. Interestingly, both

AMPK and mTOR target SREBP-1c to appropriately regulate fatty acid and triglyceride biosynthesis. PASK is proposed as a nutrient-responsive metabolic regulator. Our results show that PASK expression is induced by feeding in liver, and it regulates hepatic lipogenesis via SREBP-1c as well. Intriguingly, administration of a PASK inhibitor not only corrects hepatic and whole-body dyslipidemia, but also improves insulin sensitivity and other metabolic parameters in diet-induced obese rats, indicating that PASK is a potential therapeutic target for metabolic diseases.

While the work described in Chapter 2 has furthered our understanding of the physiological function of PASK, it has raised several interesting questions as well. For example, the mechanism(s) whereby PASK regulates SREBP-1c maturation, as well as the precise placement of PASK within the insulin-SREBP-1c regulatory pathway, still remains elusive. In the appendices, we present two preliminary studies suggesting that PASK may activate SREBP-1c maturation by directly phosphorylating the precursor form, and PASK may act as a downstream effector of mTORC1 in regulating SREBP-1 activity.

Taken together, our work suggests an overall model where nutrients and insulin signaling potentially activates PASK through mTORC1 signaling. Upon activation, PASK stimulates SREBP-1 maturation potentially via direct phosphorylation, which in turn leads to increased expression of the lipogenic enzymes and elevated lipid synthesis. As protein kinases represent one of the largest families of druggable targets, our findings provide a potential therapeutic approach to the treatment of metabolic diseases.

APPENDIX A

PHOSPHORYLATION OF SREBP-1C BY PASK – A POTENTIAL REGULATORY MECHANISM FOR SREBP-1C MATURATION

Sterol regulatory element binding protein 1c (SREBP-1c) is a transcription factor that plays crucial roles in regulating lipid metabolism in liver (Horton et al., 2002). Upon activation by feeding and insulin signaling, SREBP-1c stimulates the transcription of genes involved in fatty acid and triglyceride biosynthesis, thus promoting the conversion of dietary nutrients into lipid for storage (Xu et al., 2013). Hyperactivation of SREBP-1c has been shown to play a causal role during the development of various metabolic disorders, such as hepatic steatosis, hypertriglyceridemia and insulin resistance (Brown and Goldstein, 2008).

As the master transcriptional regulator of the lipogenic program, SREBP-1c is tightly regulated at multiple levels. One unique posttranslational regulatory step is the proteolytic maturation of the membrane-bound precursor SREBP-1c into its transcriptionally active mature form (Xiao and Song, 2013). SREBP-1c is first synthesized as a transcriptionally inert precursor form that resides on the endoplasmic reticulum (ER) membrane. Precursor SREBP-1c forms a complex with SREBP cleavage activating protein (SCAP), and is retained in the ER by an ER membrane resident protein insulin induced gene (Insig). Upon stimulation, Insig protein dissociates from SCAP, which allows SCAP to interact with the COPII trafficking machinery. As a result, precursor SREBP-1c migrates to the Golgi membrane, where it undergoes proteolytic cleavages to liberate the N-terminal transcriptionally active fragment of the protein known as mature SREBP-1c. Mature SREBP-1c then enters the nucleus to activate the transcription of its lipogenic target genes.

SREBP-1c maturation is strongly stimulated by feeding and insulin

signaling (Ye and DeBose-Boyd, 2011). This process is primarily mediated by the canonical phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway, and is partially dependent on mechanistic target of rapamycin complex 1 (mTORC1), a major downstream effector of Akt (Porstmann et al., 2008; Yecies et al., 2011). Alternatively, Akt can also activate SREBP-1c maturation in an mTORC1-independent manner (Yecies et al., 2011). Akt suppresses the *Insig2a* mRNA level by promoting its degradation via the 3' untranslated region (3' UTR), resulting in enhanced SREBP-1 maturation (Yellaturu et al., 2009b). Additionally, Akt also induces serine and threonine phosphorylation of precursor SREBP-1c (Yellaturu et al., 2009a). This phosphorylation event causes the SCAP/SREBP-1c complex to associate with the COPII trafficking machinery with higher affinity, thus promoting its ER-to-Golgi translocation and proteolytic maturation. The detailed mechanisms whereby insulin/Akt promotes *Insig2a* downregulation and SREBP-1c phosphorylation, however, remain poorly understood.

PAS kinase (PASK) is an evolutionally conserved serine/threonine kinase that functions as a metabolic regulator in response to nutrient and other signals (Hao and Rutter, 2008). Previously we have shown that PASK promotes hepatic lipogenesis in response to feeding and insulin signaling by activating SREBP-1c maturation (Wu et al., 2014). However, the detailed mechanism whereby PASK regulates SREBP-1c maturation remains unclear. Since the effect of PASK on SREBP-1c maturation is downstream of insulin signaling, we decided to examine the involvement of PASK in the known regulatory pathways linking insulin and SREBP-1c maturation.

As mentioned earlier, downregulation of *Insig2a* is required for the induction of SREBP-1c maturation upon insulin stimulation (Yecies et al., 2011; Yellaturu et al., 2009b). Previously we have observed that PASK depletion had no effect on *Insig2a* mRNA levels, suggesting that PASK does not regulate SREBP-1c maturation by suppressing *Insig2a*. However, the *Insig2a* mRNA level does not always correlate with the Insig2 protein level, as evidenced by the downregulation of *Insig2a* transcripts, but accumulation of Insig2 protein in livers from liver-specific insulin receptor knockout (LIRKO) mice upon feeding (Haas et al., 2012). To determine if PASK can regulate Insig2 at the protein level, we examined the abundance of Insig2 protein upon PASK inhibition in cultured cells and *in vivo*. No significant changes in the Insig2 protein level were observed in *PASK*-silenced HepG2 cells (Figure A-1A) or liver from rats treated with PASK inhibitor BioE-1115 (Figure A-1B), although both of these treatments suppress SREBP-1 maturation (Wu et al., 2014). These results further support the notion that the effect of PASK on SREBP-1 maturation is not mediated by Insig2a.

In addition to modulating the Insig2a level, insulin can also stimulate SREBP-1c maturation by promoting its phosphorylation and association with COPII vesicles (Yellaturu et al., 2009a). The kinase that is responsible for this phosphorylation has not been identified. Because of the serine/threonine kinase activity of PASK, as well as the presence of the PASK consensus phosphorylation sequence in SREBP-1c, we hypothesized that PASK might

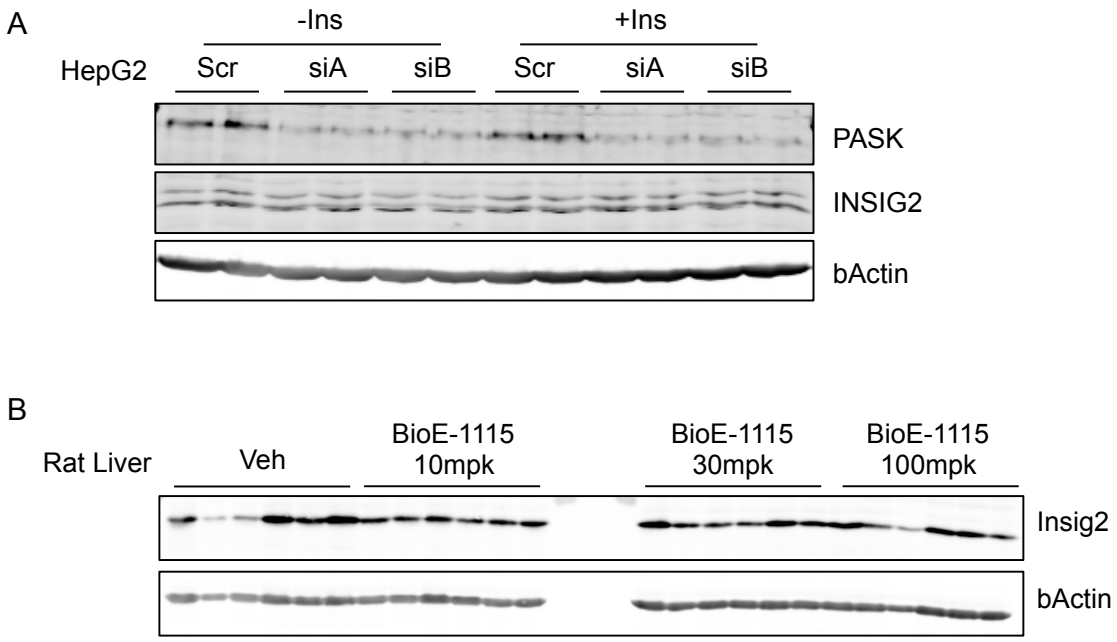

Figure A-1. PASK inhibition has no effect on the Insig2 protein level. (A) HepG2 cells were treated with scrambled or *PASK*-specific siRNA (siA or siB). Cells were then serum starved overnight and stimulated with 100nM insulin for 6 h, as indicated. Whole-cell lysates were subjected to immunoblot to determine the abundance of the indicated proteins. (B) Rats were maintained on high-fat and high-fructose diet for 18 weeks, and were subjected to once/day treatment with vehicle or the indicated dose of BioE-1115 by oral gavage for the last 3 weeks. Following this regimen, rats were fasted for 24 h and then refed for 12 h. Livers were harvested from these animals, and whole liver lysate were subjected to immunoblot for the abundance of the indicated control proteins.

phosphorylate SREBP-1c to stimulate its maturation in response to insulin signaling. To test this hypothesis, we first examined whether PASK could physically associate with SREBP-1c. As shown in Figure A-2, endogenous PASK was co-immunoprecipitated with Flag-tagged precursor SREBP-1c as well as mature SREBP-1c, although to a lesser extent. This preference for the precursor form in PASK interaction is interesting given its function in regulating SREBP-1c maturation. We then determined if PASK could directly phosphorylate SREBP-1c by performing an *in vitro* kinase assay. PASK purified from baculovirus-infected insect cells was incubated with recombinant mature SREBP-1c purified from transfected HEK293T cells in the presence of ATP. As shown in Figure A-3, mature SREBP-1c was efficiently phosphorylated by PASK. This phosphorylation was abolished by adding a PASK-specific inhibitor BioE-1197 to the reaction. These results indicate that SREBP-1c is a direct substrate of PASK *in vitro*.

In summary, here, we have presented preliminary data suggesting that upon insulin stimulation PASK activates SREBP-1c maturation, potentially by direct phosphorylation. Phosphorylation of SREBP-1c has been demonstrated as an important regulatory mechanism for its maturation. For example, AMP-activated protein kinase (AMPK) phosphorylates mature SREBP-1c at serine residue 372 (Li et al., 2011). Serine to alanine mutation of this phosphorylation site in the precursor form abolished the inhibitory effect of AMPK on SREBP-1c maturation. On the other hand, elevated serine and threonine phosphorylation of SREBP-1c induced by insulin enhances its affinity for the COPII vesicles and

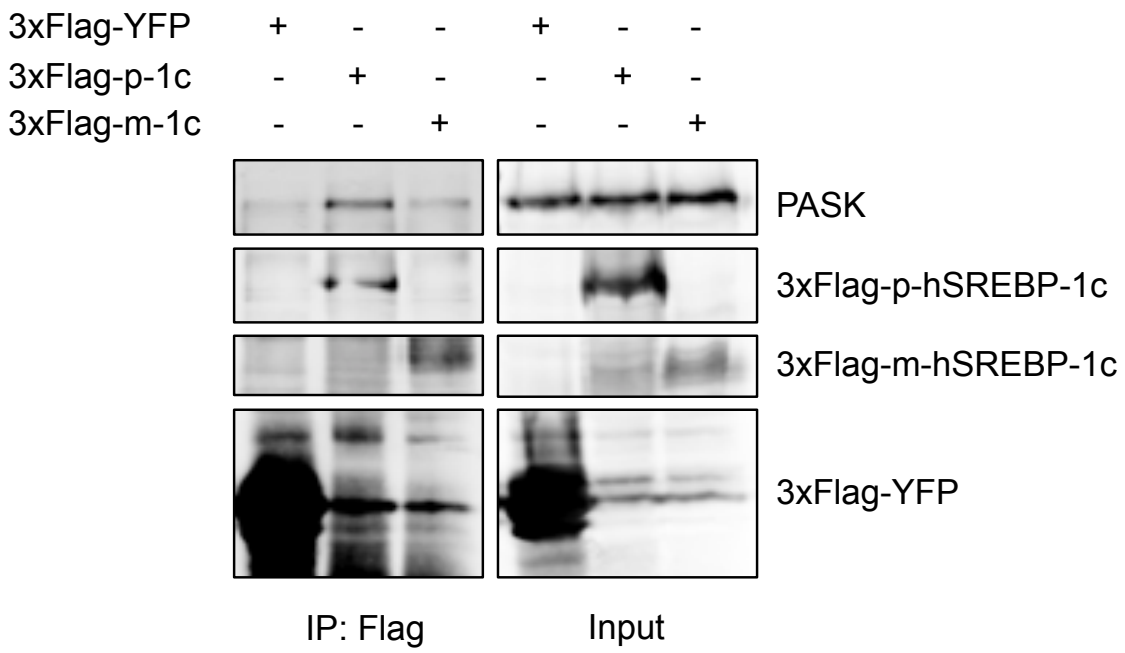

Figure A-2. PASK co-immunoprecipitates with SREBP-1c. Flag-tagged YFP, precursor or mature hSREBP-1c was expressed in HEK293T cells. Flag immunoprecipitates were subjected to immunoblot to examine the co-precipitation of PASK (Left). Input represents 5% of the total cell lysate used for immunoprecipitation (right).

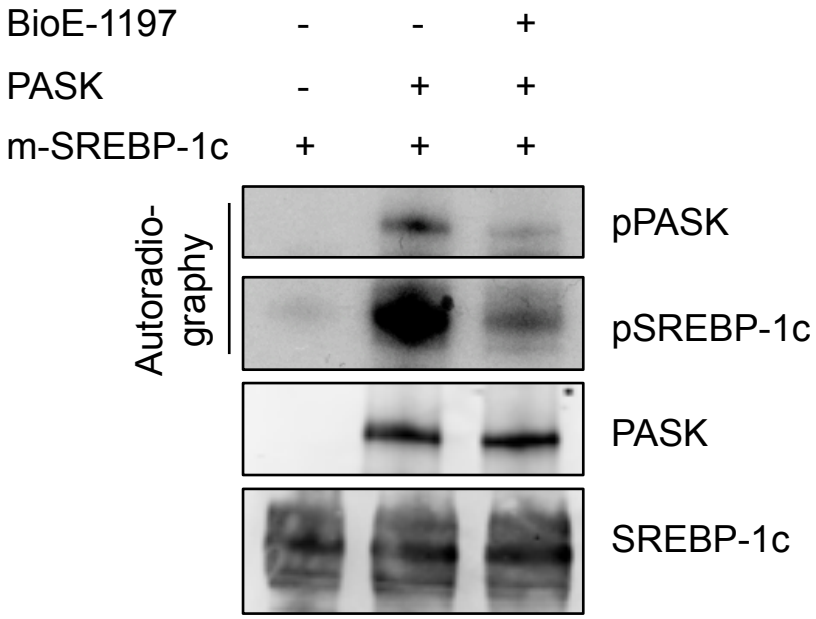

Figure A-3. PASK phosphorylates SREBP-1c *in vitro*. *In vitro* kinase assay was performed using hPASK purified from baculoviral-infected insect cells and mature hSREBP-1c purified from HEK293T cells in the presence or absence of BioE-1197. The reaction mixture was subjected to immunoblot to examine the phosphorylation state (autoradiography) and abundance of the indicated proteins.

promotes its translocation to the Golgi for proteolytic maturation (Yellaturu et al., 2009a). We showed that PASK can directly phosphorylate SREBP-1c *in vitro*. Although the mature SREBP-1c, which is the N-terminal fragment of the protein, was used in the kinase assay, it is likely that PASK can phosphorylate the precursor SREBP-1c as well. This is because the N-terminal portion of SREBP-1c is localized on the cytosolic side of the ER membrane (Brown and Goldstein, 1997; Hua et al., 1995), and is therefore accessible to PASK. Future studies will be aimed toward identifying the PASK phosphorylation site(s) on SREBP-1c, as well as examining the effect of such phosphorylation event(s) on SREBP-1c maturation.

A.1 Material and Methods

A.1.1 Animals

Sprague-Dawley (SD) male rats (Charles River Laboratories) were housed under standard vivarium conditions (12 h light/dark cycle) with water and chow ad libitum. All studies were approved by the Institutional Animal Care and Use Committee guidelines of the University of Utah and/or St. Louis University as appropriate.

Rats were maintained on high-fat and high-fructose diet for 18 weeks, and were treated with vehicle or indicated dose of BioE-1115 as previously described for the last 3 weeks (Wu et al., 2014). Animals were euthanized by CO2 asphyxiation. Liver tissue was taken during necropsy, weighed and snap frozen in liquid N_2.

A.1.2 Cell Lines and Cell Culture

HepG2 and HEK293T cells were obtained from ATCC. HepG2 cells were maintained in 1:1 DMEM/F-12 (HyClone) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma). HEK293T cells were maintained in DMEM (Mediatech) supplemented with 10% FBS and 1% penicillin/streptomycin.

A.1.3 Plasmids and Plasmid Constructions

N-terminally Flag-tagged precursor or mature SREBP-1c was cloned from human SREBP-1 cDNA (BC057388; Open Biosystems) and ligated into the pQCXIN retroviral vector to generate pQCXIN-3xFlag-p-hSREBP-1c or pQCXIN-3xFlag-m-hSREBP-1c. Both constructs were verified by sequencing. pcDNA3.1-2xFlag-mSREBP-1c, a plasmid expressing the mature form of human SREBP-1c, was obtained from Addgene (plasmid # 26802). pSRE-Luc, a plasmid containing the hamster HMG-CoA synthase sterol regulatory elements fused to luciferase cDNA, was a kind gift from Dr. Timothy F. Osborne (Sanford-Burnham Medical Research Institute).

A.1.4 PASK Silencing

HepG2 cells were transfected with scrambled (Allstars negative control siRNA, Qiagen) or human PASK-specific siRNAs (Qiagen) using HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. Cells were analyzed 96 h after transfection.

A.1.5 Immunoprecipitation and Immunoblot

For co-immunoprecipitation experiments, HEK293T cells were transfected with Flag-tagged YFP, precursor or mature hSREBP-1c using Lipofectamine 2000 (Invitrogen), according to the manufacture's instructions. Cells were then lysed in lysis buffer (40 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 2mM EGTA, 2mM KCl, 1% CHAPS and protease inhibitors). After centrifugation, Flag-tagged YFP or hSREBP-1c was immunoprecipitated using anti-Flag M2 affinity gel (Sigma-Aldrich A2220). For *in vitro* kinase assay, Flag-tagged mature hSREBP-1c was expressed and purified from HEK293T cells as described previously (Kikani et al., 2010).

For immunoblot, HepG2 cell lysates were prepared by directly dissolving cells in 1X SDS sample buffer. Liver lysate were prepared as described previously (Wu et al., 2014). Protein samples were then separated by SDS-PAGE and transferred to nitrocellulose for blocking and incubation with primary antibody and secondary antibody conjugated with infrared dyes for detection by the Odyssey system (LI-COR). Primary antibodies were obtained from the following sources: anti-hPASK (U2501) as previously described; anti-Flag (M2) and anti-bActin (A5441) from Sigma; anti-Insig2 (Ab86415) from Abcam.

A.1.6 *In Vitro* Kinase Assay

Flag-tagged mature hSREBP-1c was purified from HEK293T cells as described above. Kinase assay was performed as described previously (Kikani et al., 2010) using immuno-purified mature hSREBP-1c and hPASK purified from

baculoviral infected insect cells in the presence or absence of BioE-1197. Samples were then separated by SDS-PAGE and transferred to nitrocellulose for autoradiography and immunoblot.

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APPENDIX B

INVOLVEMENT OF PASK IN mTORC1-MEDIATED SREBP-1 ACTIVATION

Insulin is a key anabolic hormone that promotes the uptake and storage of excess nutrients in metabolic tissues, such as liver, muscle and white adipose tissue (Samuel and Shulman, 2012). In liver, the major actions of insulin are to suppress gluconeogenesis while stimulating glycogen synthesis and lipogenesis. The pro-lipogenesis effect of insulin is primarily mediated by Akt, and is dependent on sterol regulatory element binding protein 1c (SREBP-1c), the master transcriptional regulator of lipid metabolism in liver. In response to insulin signaling, SREBP-1c activates the transcription of genes involved in fatty acid and triglyceride biosynthesis, thereby promoting the production of triglyceride from dietary nutrients for energy storage.

SREBP-1c is activated by insulin/Akt at both transcriptional and posttranslational levels (Krycer et al., 2010), but the exact mechanism underlying this regulatory effect is not completely understood yet. Growing evidence indicates that mechanistic target of rapamycin complex 1 (mTORC1), a protein kinase complex activated by Akt, plays an essential role in this pathway. Upon activation, mTORC1 promotes (1) the transcription of *SREBP-1c* (Li et al., 2010); (2) the posttranslational proteolytic maturation of precursor SREBP-1 to generate the transcriptionally active mature form of the protein (Owen et al., 2012; Porstmann et al., 2008; Yecies et al., 2011); and (3) the accumulation of mature SREBP-1 in the nucleus (Peterson et al., 2011). Genetic and pharmacological inhibition of mTORC1 suppressed SREBP-1 activity and lipogenesis both *in vitro* and *in vivo* (Owen et al., 2012; Peterson et al., 2011; Porstmann et al., 2008; Yecies et al., 2011). The mechanisms whereby mTORC1 activates SREBP-1

remain largely unclear, and only few downstream effectors have been identified. p70 ribosomal S6 kinase (S6K), a kinase directly activated by mTORC1, has been reported to mediate mTORC1's effect on SREBP-1 maturation under some but not all conditions (Duvel et al., 2010; Lewis et al., 2011; Owen et al., 2012), suggesting an alternative mechanism distinct from S6K activation.

We have previously shown that PASK activates SREBP-1 by promoting its maturation in response to insulin signaling (Wu et al., 2014). However, the precise placement of PASK within the insulin-SREBP-1c regulatory pathway is still not clear. Interestingly, unpublished data from our lab indicate that PASK activity is induced by insulin stimulation in an mTORC1-dependent manner. Moreover, mTORC1 directly binds PASK and phosphorylates it at multiple sites to promote its activity. These results raise a hypothesis that in response to insulin signaling, PASK may function downstream of mTORC1 to activate SREBP-1.

To test this hypothesis, we first sought to determine whether PASK acted in the same pathway as mTORC1 to regulate SREBP-1. To test this, we examined the individual and combined effects of PASK silencing and mTORC1 inhibition by rapamycin on insulin-induced SREBP-1 activation in HepG2 cells. SREBP-1 activity was measured using a luciferase reporter that contains isolated SREBP binding sites in its promoter region. Consistent with our previous observation, insulin treatment caused a significant increase in SREBP-1 activity in control cells (Figure B-1). This increase was partially or completely suppressed by PASK knockdown or rapamycin treatment, indicating that inhibition of PASK or mTORC1 alone is sufficient to prevent insulin-stimulated SREBP-1 activation.

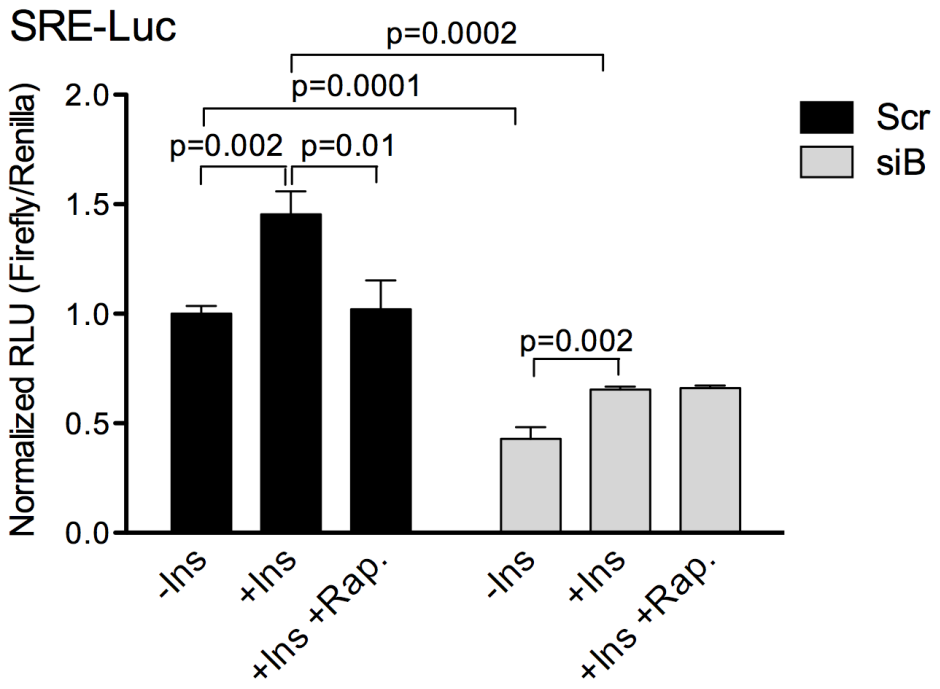

Figure B-1. PASK and mTORC1 function in a linear pathway to activate SREBP-1. HepG2 cells were treated with scrambled or *PASK*-specific siRNA (siB), and then transfected with SRE-Luc reporter. Cells were serum starved overnight in the presence or absence of rapamycin, and then stimulated with 100nM insulin for 6 h, as indicated. Firefly and Renilla luciferase were assayed using the Dual-Reporter Luciferase Assay System. Data shown are the average of n=3 ± SD, with the “Scr -ins” value set as 1.

Interestingly, combined inhibition of PASK and mTORC1 did not further reduce SREBP-1 activity compared to PASK silencing alone, suggesting that PASK and mTORC1 function in a linear pathway to regulate SREBP-1 activation.

To directly test whether PASK is an effector downstream of mTORC1 in regulating SREBP-1 activity, we examined the effect of PASK inhibition on SREBP-1 activity upon mTORC1 activation. As shown in Figure B-2, overexpression of Rheb, a small GTPase that activates mTORC1 (Sarbassov et al., 2005), greatly induced SREBP-1 activity. This induction was reduced by 60% in PASK inhibitor BioE1197-treated cells, indicating that PASK is required for the full activation of SREBP-1 induced by mTORC1.

Our preliminary results suggest that PASK is a major downstream effector of mTORC1 in activating SREBP-1. mTORC1 activates SREBP-1 at multiple levels (Xu et al., 2013). Given the critical role of PASK in regulating SREBP-1 maturation, it is conceivable that PASK may mediate the effect of mTORC1 on SREBP-1 maturation. Further studies will need to be done to directly examine SREBP-1 maturation upon manipulation of PASK and mTORC1.

Hyperactivation of SREBP-1c lies at the heart of a pathological condition in liver called “selective insulin resistance” (Brown and Goldstein, 2008). Under this condition, insulin fails to activate glycogen synthesis and suppress gluconeogenesis in liver due to impaired insulin signaling. However, the SREBP-1c-mediated lipogenesis remains fully active, which leads to the combination of hyperglycemia and hypertriglyceridemia observed in diabetic patients. One potential explanation of this paradox is that SREBP-1c may be preferentially

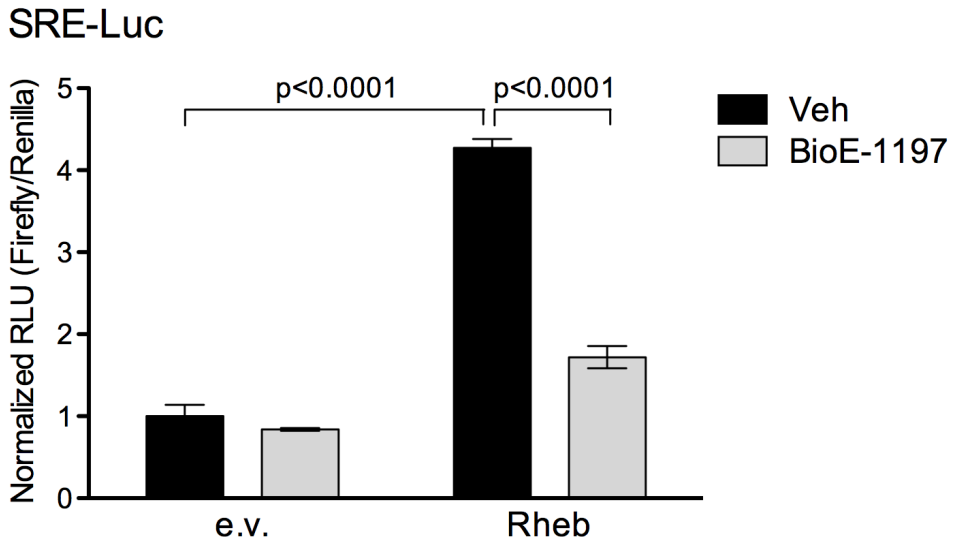

Figure B-2. PASK is a downstream effector of mTORC1 in regulating SREBP-1. HepG2 cells were transfected with empty vector or wild-type Rheb along with SRE-Luc reporter. Cells were then treated overnight with vehicle or 50 μM BioE-1197 in media with 2% FBS, as indicated. Firefly and Renilla luciferase were assayed using the Dual-Reporter Luciferase Assay System. Data shown are the average of n=3 ± SD, with the “e.v. +Veh” value set as 1.

activated by stimuli other than insulin. It is well known that, in addition to insulin, mTORC1 can also be activated by glucose and amino acids, two nutrients that are highly abundant under insulin resistant conditions (Zoncu et al., 2011). Moreover, mTORC1 has been shown to selectively regulate the expression of SREBP-1c and its lipogenic target genes in liver, while having no effect on the expression of gluconeogenic genes (Li et al., 2010). Therefore, it is possible that under insulin resistant conditions, excess nutrients lead to the continuous activation of mTORC1, which in turn promotes SREBP-1c activity and lipogenesis, eventually resulting in hepatic and whole-body dyslipidemia. Our data suggest that PASK is a new link in the mTORC1-SREBP-1 pathway, and may play a role in promoting the development of selective insulin resistance in liver.

In addition to metabolic diseases, aberrant activation of mTORC1 has also been connected to the initiation and progression of cancer (Zoncu et al., 2011). The effect of mTORC1 on tumorigenesis is partly attributed to its ability to promote metabolic reprogramming of cancer cells to support their rapid growth and proliferation (Duvel et al., 2010). One important aspect of such reprogramming is elevated lipogenesis due to the increased demand for membrane synthesis during cell growth and proliferation. Hyperactivation of mTORC1 has been shown to render tumor cells addicted to exogenous lipids under hypoxic conditions where *de novo* lipogenesis is reduced (Young et al., 2013). Interestingly, it has been demonstrated that SREBP-1c as well as its lipogenic target genes, including acetyl-CoA carboxylase 1 (*ACC1*) and fatty acid

synthase (*FASN*), are upregulated in a wide range of cancers, and are required for cancer cell growth and survival (Guo et al., 2014). Since PASK is potentially an intermediate regulator in the mTORC1-SREBP-1c pathway, it is tempting to speculate that PASK inhibition may prove valuable for cancer treatment.

B.1 Material and methods

B.1.1 Cell Lines and Cell Culture

HepG2 and HEK293T cells were obtained from ATCC. HepG2 cells were maintained in 1:1 DMEM/F-12 (HyClone) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma).

B.1.2 Plasmids

pHAGE-CMV-Rheb-IRES-eGFP-W, a plasmid expressing wild-type human Rheb, was obtained from Addgene (Plasmid #32519). pSRE-Luc, a plasmid containing the hamster HMG-CoA synthase sterol regulatory elements fused to luciferase cDNA, was a kind gift from Dr. Timothy F. Osborne (Sanford-Burnham Medical Research Institute).

B.1.3 PASK Silencing

HepG2 cells were transfected with scrambled (Allstars negative control siRNA, Qiagen) or human PASK-specific siRNAs (Qiagen) using HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. Cells were analyzed 96 h after transfection.

B.1.4 Luciferase Assay

HepG2 cells were cotransfected with (1) pSRE-Luc; (2) a construct expressing CMV-driven Renilla luciferase (Promega); (3) pHAGE-CMV-Rheb-IRES-eGFP-W construct, as indicated, using Lipofectamine LTX (Invitrogen), according to the manufacture's instructions. After transfection, cells were (1) serum-starved overnight in the presence of 20 nM rapamycin, followed by 100 nM insulin for 6 h before harvest; or (2) treated overnight with vehicle or 50 μM BioE-1197 in DMEM/F-12 with 2% FBS. Firefly and Renilla luciferase were assayed using the Dual-Reporter Luciferase Assay System (Promega).

B.1.5 Statistical Analysis

Data are presented as mean ± standard deviation unless otherwise indicated. A two-tailed equal variance t-test was used to compare differences, and the null hypothesis was rejected at the 0.05 level.

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